EXHIBIT 9

Elimination of infectious retroviruses during preparation of immunoglobulins

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ABSTRACT. Safety concerns for immunoglobulin preparations have led us to study partition/inactivation of two prototype retroviruses, mouse xenotropic type C and lymphadenopathy-associated virus (LAV) of the acquired immunodeficiency syndrome (AIDS), during manufacture and storage of immunoglobulins. Reduction of infectious retrovirus titers were 10^5 to 10^6-fold through Cohn-Oncley cold ethanol fractionation from plasma to fraction II, 10^3 to 10^5-fold through incubation at pH 4.0 and another 10^4-fold through incubation of the purified liquid immunoglobulin preparations at 27°C or 45°C. The results support the clinical and epidemiological evidence that therapeutic immunoglobulin preparations do not transmit AIDS virus.

Antibodies to the retrovirus associated with the acquired immunodeficiency syndrome (AIDS) have been detected in human hepatitis B immunoglobulin (HBIG) (1) as well as in other commercial lots of immunoglobulins (2). This observation raised the possibility that immunoglobulin products transmit infectious virus. This concern was heightened by recent reports of non A, non B (NANB) hepatitis in immunodeficient patients who had received infusions of intravenous immunoglobulins prepared from Cohn fraction II (3,4).

Based on the above findings, we determined the ability of retroviruses to withstand the procedures employed in manufacturing immunoglobulin preparations. For these experiments, two prototype retroviruses were used: the mouse xenotropic type C retrovirus and the LAV strain of the AIDS retrovirus.

Materials and Methods

The mouse xenotropic type C retrovirus recovered from a New Zealand Black mouse kidney was grown in mink lung cells (5). Detection was based on a focus assay in mink S+L- cells (6) and titer determined by the induction of the viral core structure protein (p30) measured by immunofluorescence (7). The assays were used previously in mouse C virus spiking experiments with plasma fractions (8,9).

Lymphadenopathy-associated virus (LAV) was cultured and obtained from the Centers for Disease Control (CDC) in Atlanta. Its detection was based on a sandwich enzyme-linked immunoassay (ELISA) previously described (10).

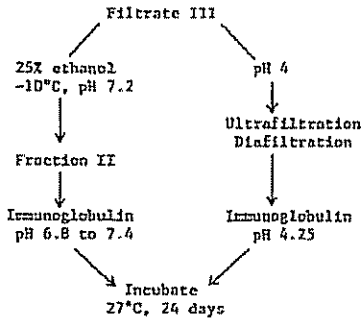

Fig. 1. Processing of Filtrate III to immunoglobulin preparations: ultrafiltration/diafiltration, pH 4 and precipitation of fraction II.

Human plasma samples were spiked with retroviral preparations and fractionated according to classical Cohn-Oncley cold ethanol procedures (11,12) through selective precipitations in the cold at various ethanol concentrations and pH values: fraction I at 8 percent ethanol, -2°C, pH 7.4; fraction II+III at 21 percent ethanol, -5°C, pH 6.7; fraction II+IIIw at 20 percent ethanol, -5°C, pH 6.5; fraction III at 18 percent ethanol, -6°C, pH 5.4; and fraction II collected at 25 percent ethanol, -10°C, pH 7.2. Residual retroviral levels were determined across the fractionation steps. Immunoglobulin preparations comparable to therapeutically used products were obtained from filtrate III by adjusting to pH 4 followed by ultrafiltration/diafiltration or by reconstituting the precipitated fraction II at neutral pH (see Figure 1). Filtrate III was also used to study the effects on virus infectivity of pH (range 5.4 to 4.0) and temperature (range -5 to 22°C) in the presence of 18 percent ethanol.

In separate experiments, final container liquid immunoglobulin products, free of ethanol, were incubated with retrovirus concentrates at 27 and 45°C; virus infectivity was determined at different time periods.

Results

Infectivity of both the mouse and LAV was not affected by the addition of these viruses to human plasma at 5°C (Table 1). From plasma to fraction II+IIIw, no more than a 10-fold reduction of virus titer was observed. Preparation of filtrate III

Table 1. Effect of immunoglobulin fractionation procedures on infectious retrovirus added to plasma*

Sample	House Type C (Total IP)	AIDS Virus (Total ID_{50})
Virus alone	2.0×10^8	2.3×10^5
Virus + plasma (5°C)	2.3×10^8	4.4×10^5
II+IIIw	3.8×10^7	4.8×10^4
Filtrate III	1.6×10^3	1.7×10^3
Fraction II	Not detectable	Not detectable

* Twenty ml of virus concentrate was added to 200 ml of plasma for the fractionation studies. The fractionation methods and viral assays are described in the text. Total IP = total infectious particles. Total ID_{50} = ID_{50} per ml (reciprocal of dilution at which 50% of the cultures are positive) x volume.

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Table 2. Effect of pH and temperature on mouse type C retrovirus added to filtrate III (18 percent ethanol)

Sample	Temperature −5°C (a) pH 5.4 (Total IP)*	(b) pH 4.7 (Total IP)	(c) pH 4.0 (Total IP)	Sample	Temperature 22°C (d) pH 5.4 (Total IP)	(e) pH 4.0 (Total IP)
Virus alone	7.9×10^6	7.9×10^6	7.9×10^6	Virus alone	4.0×10^8	5.0×10^7
Virus + filtrate III	2.9×10^6	3.4×10^5	6.5×10^5	Virus + filtrate III	2.2×10^9	5.5×10^5
2 hours	3.4×10^5	6.6×10^6	2.0×10^5	3 hours	2.2×10^8	Not detectable
4 hours	6.7×10^5	6.7×10^5	2.6×10^5			
6 hours	7.8×10^5	6.6×10^5	4.1×10^5			

* Total infectious particles were detected as described under Materials and Methods. Detection limit approximately $10^{-0.2}$ IP/ml. Two ml of mouse type C virus concentrate was added to 20 ml of effluent III for each of (a), (b), and (c). Ten ml and 5 ml of mouse type C virus were added to 100 ml and 50 ml of effluent III, respectively for (d) and (e).

Table 3. Effect of pH and temperature on LAV added to final container liquid immunoglobulin preparations

Sample	Temperature 27°C pH 6.8 IgG (Total ID_{50})*	pH 4.25 IgG (Total ID_{50})	Sample	Temperature 45°C pH 6.8 IgG (Total ID_{50})
Virus + IgG	1.65×10^4	3.69×10^3	Virus + IgG	1.65×10^4
3 days	Not detectable	Not detectable	1 hour	6.27×10^3
12 days	Not detectable	Not detectable	4 hours	1.65×10^3
24 days	Not detectable	Not detectable	8 hours	Not detectable
			20 hours	Not detectable

* ID_{50} of LAV as defined in Table 1. ID_{50} detection limit $10^{1.0}$. 1.5 ml of an LAV preparation was added to 15 ml of IgG solution for each of the two parts of the experiment.

from fraction II+III$_W$ resulted in an approximately 10,000-fold reduction of the mouse type C retrovirus and 10-fold reduction in LAV. Due to dilution, ethanol concentration decreased from 20 percent v/v to 18 percent v/v across this fractionation step and the pH was reduced from 6.50 to 5.40. Fraction II precipitation from filtrate III resulted in greater than 1000-fold reduction in titer of both the infectious mouse and human retroviruses. During this fractionation step, the pH was raised to 7.25 and the ethanol concentration increased to 25 percent. The 1000-fold loss of virus infectivity results primarily from virus inactivation (not fractionation) since after extensive dialysis, no infectious virus was measurable in the supernatant corresponding to fraction II (data not shown).

In studying more precisely the effect of pH and temperature on retrovirus inactivation with 18 percent ethanol, we mixed a quantity of the mouse retrovirus with filtrate III (Table 2). At −5°C, no significant virucidal effect was seen in the pH range of 5.4 to 4.0 for up to 6 hours (Table 2, a, b, c). However, at 22°C (ambient) and at pH 4.0 greater than 100,000 infectious mouse retrovirus particles were inactivated by 3 hours (Table 2, e). In contrast, at pH 5.4 under similar conditions (Table 2, d), no significant virucidal effect was seen. Similarly, 1.7×10^3 total ID_{50} of LAV that was in a filtrate III solution at pH 4.0 and held at +5°C for 18 hours, was reduced to a non-detectable level (data not shown). It appears that the presence of 18 percent ethanol in plasma fractions at a pH of 5.4 was not markedly virucidal for these viruses in the temperature range of −5 to 22°C. Significant virus inactivation was observed only when the pH was lowered (pH 4.0) concomitant with a rise in temperature (≥5°C). Under these conditions and at 18 hours, a 10^4-fold reduction of LAV (data not shown) and a greater than 10^7-fold reduction in mouse type C retrovirus was measured (Table 2). To determine the effect on LAV of the pH and temperature in the final product, final container liquid immunoglobulin preparations (protein concentration 5 percent w/v) were incubated with LAV (Table 3). At 27°C, between 10^3 to 10^4 of total ID_{50} were inactivated within 3 days for the immunoglobulin preparations of both pH 6.8 and pH 4.25. At 45°C, greater than 10^4 total ID_{50} were inactivated within 8 hours with the pH 6.8 immunoglobulin preparation. The pH 4.25 immunoglobulin preparation was not tested at 45°C.

Discussion

These experiments were conducted to evaluate the effect on infectious retroviruses of procedures used for immunoglobulin manufacture. These included: Cohn-Oncley fractionation, temperature and pH, and liquid incubation. The data are important in assessing the possible risk of LAV contamination of some Ig preparations. The mouse type C retrovirus was used as well as the LAV strain of AIDS virus, because the former can be grown to very high titer and therefore the effect of various procedures can be more precisely quantitated by a focus assay.

Unlike the reported complement-mediated lysis of many retroviruses by human serum at 37°C (13), LAV in the cold (0 to 5°C) was not affected by this mechanism (14). The 10^5-fold reduction of mouse type C virus and 10^2-fold reduction of LAV from plasma to filtrate III was primarily due to partitioning effects, since data reported in Table 2 demonstrate that ethanol (18 percent) in the cold (−5°C) and in the presence of plasma fractions was not strongly virucidal for mouse type C virus. The reported virucidal effects of ethanol for LAV have been at ambient temperature (15,16). The difference in reduction between the mouse and the

human virus reflects either a greater resistance of LAV to the processing conditions or a less quantitative assay for this virus. As noted above, the mouse virus can be grown to high titers and its assay is very reproducible. Its usefulness for fractionation/inactivation studies has been reported previously (8,9).

Ethanol concentration is increased to 25 percent v/v at pH 7.2 for the fraction II precipitation which resulted in a more than 1000-fold inactivation of the mouse type C virus and LAV. Since the corresponding effluent was free of infectious virus, true inactivation at the 25 percent ethanol concentration is most likely involved. Our results are in agreement with those of Wells et al. (17) who also noted that 25 percent ethanol in plasma fractions was strongly virucidal for HTLV-III at −5°C. A recent report (18) showed inactivation of $10^{4.5}$ ID_{50} of LAV during the precipitation of I+II+III (20 percent ethanol v/v, pH 6.9, temperature −5°C) under conditions in which fraction II+III is precipitated together with fraction I. Our results which isolated these fractions separately did not show such complete LAV inactivation under similar conditions (Table 1). In our study, the samples were extensively dialyzed in phosphate buffer saline prior to ID_{50} assay. In the other report, a 1:10 dilution to a resultant residual ethanol concentration of 2 percent v/v was used in the assay. Furthermore, it is not possible from the other report to distinguish whether the virus titer was being determined in the precipitate or the supernatant following I+II+III precipitation; hence, a meaningful comparison between the two studies is difficult to make.

Filtrate III with 18 percent ethanol at pH 5.4 and at a temperature of −5°C was not significantly virucidal for retroviruses for extended periods of time. Enhanced inactivation at pH 4.0 was strongly dependent on temperature both for mouse C virus (Table 2) and for LAV. In the latter case, a 1000-fold reduction of virus titer was noted following incubation at +5°C for 18 hours under modified filtrate III conditions (pH 4.0, ethanol 18 percent).

Greater than a 1000-fold drop in LAV infectivity did result after its incubation with purified liquid immunoglobulin preparations at 27°C for 3 days; the pH of the purified immunoglobulin preparations did not seem to have an appreciable effect. A higher incubation temperature (45°C) demonstrated comparable titer reduction within 8 hours. A "worse case" estimate of 2000 ID per ml of LAV in large plasma pools has been reported (19). The yield of IgG could be as low as 50 percent of the amount present in plasma together with an IgG concentration increase from approximately 1 gm per 100 ml in plasma to 5 gm per 100 ml in the purified product. If the AIDS virus was concentrated without loss of infectivity along with IgG purification, the purified IgG would contain 2000 ID per ml x 10 or 2×10^4 ID per ml. Immunoglobulin purification processes must therefore be able to fractionate/inactivate 2×10^4 ID per ml of AIDS virus.

No single step in the Cohn cold ethanol process can completely inactivate retroviruses. However, the effects of fractionation and inactivation taken together through the fractionation cascade could be quite large. Indeed, the communication by Wells et al. (17) in which plasma was fractionated by ethanol and the effectiveness of HTLV-III elimination determined at each of six steps demonstrated a cumulative efficiency of the entire process to be such that infectivity was reduced by more than 1×10^{15} infectious units per ml. In the present study, LAV recovery from plasma to fraction II was reduced by at least 100,000-fold; pH adjustment to 4.0 at filtrate III (at +5°C) was as effective for viral inactivation as precipitation of fraction II in the presence of 25 percent ethanol. An extra margin of safety was provided when the final preparation in liquid form was incubated at 27°C; and, under these conditions, we demonstrated that in liquid immunoglobulin preparations, a 1000 to 10,000-fold reduction of LAV occurred within 3 days. Prince et al. (20) have suggested that the long storage of liquid immune serum globulin preparations may contribute to their safety. The studies presented here experimentally validate that LAV was inactivated during liquid storage (Table 3). The chance for an infectious retrovirus to survive this fractionation as well as storage of the liquid final preparation probably is extremely small.

The fractionation/inactivation and final container incubation results reported here support the available clinical and epidemiological evidence that therapeutic immunoglobulins prepared by the Cohn-Oncley cold ethanol process do not transmit AIDS viruses. The conditions of the Cohn-Oncley process i.e., alcohol concentration, pH, temperature, do not in themselves inactivate AIDS virus as recently reported by Prince et al. (20). As described, their study was primarily designed to determine inactivation, and no sequential fractionation was carried out. In contrast, the present study mimicked a true fractionation run and hence portrayed a realistic estimate of virus carryover involving the sum total of fractionation and inactivation.

It is important to emphasize that variations from the classical Cohn approach need to be validated in terms of their virucidal and virus distribution potential since fractionation, ethanol concentration, pH, and temperature all play an important role in virus recovery. It is possible that total log reduction of different viruses could be different and hence it would be difficult to generalize these virus recovery results for other viruses.

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EXHIBIT 10

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Inactivation of Hepatitis C Virus in Low pH Intravenous Immunoglobulin

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Abstract. The safety from hepatitis C virus of intravenous immunoglobulin prepared by the cold ethanol method of Cohn-Oncley is demonstrated by clearance through the manufacturing process of 9×10^6 plaque-forming units of bovine viral diarrhea virus used as a surrogate for hepatitis C virus. Incubation of the intravenous immunoglobulin in its final formulation at pH 4.25 for 21 days at 21°C caused a 10 000-fold decrease in bovine viral diarrhea virus intentionally added and complete inactivation of 1000 chimpanzee infectious doses per ml of hepatitis C virus.

Introduction

Published reports[1–5] indicate that immunoglobulins for intravenous administration (IGIV) should be safe with respect to transmission of viral infections. However, occasional reports of hepatitis C transmission by these products[6–10] have prompted an examination of the manufacturing process for clearance and/or inactivation of hepatitis C virus (HCV) and incorporation in the process of a definitive virus inactivation step.

In this report we used bovine viral diarrhea virus (BVDV) of the pestivirus group as a surrogate for HCV in those studies requiring virus infectivity titration of numerous samples thereby making impractical the use of HCV which can only be tested in chimpanzees and marmosets. BVDV was shown to be cleared through the immunoglobulin fractionation process; its inactivation in IGIV was demonstrated to be pH dependent. In a definitive experiment, HCV added to IGIV at pH 4.25 was shown to be inactivated as tested by inoculation of chimpanzees for detection of residual live virus.

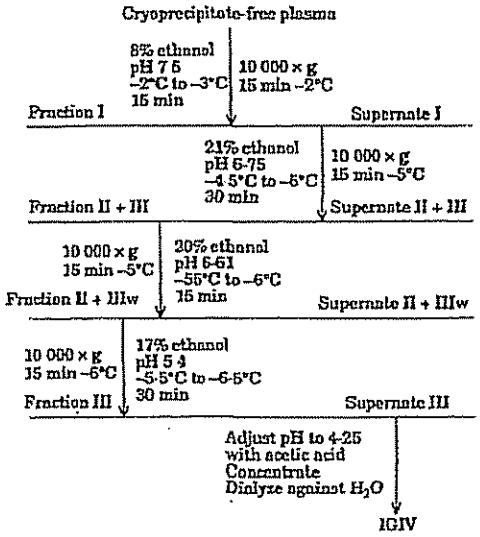

Figure 1. Flow diagram for fractionation of IGIV from cryoprecipitate-free plasma by the method of Cohn and Oncley. Alcohol concentrations, pH and temperature of each step are as indicated.

Materials and methods

IGIV preparation

Plasma fractionation was performed by a modified Cohn[11] and Oncley[12] method adapted as necessary for laboratory scale (Fig. 1). Frozen normal human plasma was thawed at 4°C and centrifuged at 16 000 *g*, 4°C for 25 min to remove the cryoprecipitate. To 100 ml of the supernatant cryoprecipitate-free plasma, BVDV was added to a final concentration of 9×10^4 pfu/ml. The plasma was then adjusted to pH 7.5, 95% ethanol was added to 8% while stirring and cooling to between −2° and −3°C. The suspension was held at this temperature for 15 min and then centrifuged at 10 000 *g*, −2°C for 15 min. The fraction I precipitate was collected, supernate I was adjusted to pH 6.75, the ethanol was

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adjusted to 21%, the temperature was lowered to between −4·5° and −5°C and stirred for 30 min. The suspension was centrifuged at 10 000 *g*, −5°C for 15 min. Supernate II + III was collected and the precipitated fraction II + III was suspended in 66·2 ml of ice water. The mixture was stirred while the pH was adjusted to 6·61; ethanol was added to 20% while the temperature was adjusted to between −5° and −6°C. After stirring for 15 min, the suspension was centrifuged at 10 000 *g*, −5°C for 15 minutes. The supernate II + IIIw was collected. Precipitated fraction II + IIIw was suspended in 85 ml of ice water and stirred for 15 min at 1°C. The pH was adjusted to 5·4 and ethanol was added to 17% while cooling to between −5·5° and −6·5°C. After stirring under these conditions for 30 min the suspension was centrifuged at 10 000 *g*, −6°C for 15 min. The precipitated fraction III was collected. The final form of IGIV is derived from supernate III by adjusting the solution to pH 4·25 with acetic acid, concentrating by ultrafiltration, dialyzing against water to remove ethanol, and adjusting the protein concentration to 5%.

Viruses

The Hutchinson strain of HCV was obtained from Dr R. Purcell of the National Institutes of Health, Bethesda, MD, U.S.A. An RNA transcript representing the approximate first 400 nucleotides of the HCV genome was supplied by Dr S. Feinstone, NIH. The NADL strain of BVDV was obtained from Dr J. Polley of TSI Washington Laboratories, Kensington, MD, U.S.A. Plasmid pBV-KPB containing nucleotides 9835 to 12225 of BVDV was obtained from Dr M. Collett of Medimmune, Inc, Gaithersburg, MD, U.S.A.

BVDV plaque assay

Bovine turbinate cells (CRL1390) obtained from ATCC were used for the BVDV plaque assay. Confluent cell monolayers grown in Eagle's minimal essential medium supplemented with non-essential amino acids, 1 mM pyruvate, 10% equine serum and 50 μg/ml of gentamicin in six-well plates were inoculated with diluted samples of BVDV, two wells were used per dilution. The virus was allowed to adsorb to the cells for 1 h at 37°C and then removed. The cells were overlaid with medium containing 0·7% agarose, incubated for 5 to 7 days and then stained with 0·3 g/l neutral red in growth medium for enumeration of plaques. Monolayers were monitored for ethanol toxicity 24 hours after inoculation. Any well showing toxicity was removed from the study.

Detection of BVDV RNA by polymerase chain reaction (PCR)

Two primers which amplified a 205 bp fragment between nucleotides 9893 and 10098 of the viral genome were used.[13] The sequence of the primers was 5′-GATTT CAAGG GGACT TTTTT (primer 2.1) and 5′-ACATC TCCTA CTAAG TAGTA (primer 2.2). To extract RNA from the samples, 100 μl was combined with 300 μl of 50 mM Tris-HCL, 5 mM EDTA, 0·25% SDS, 150 mM NaCl, 1 mg/ml proteinase-K, pH 7·5, and incubated at 50°C for 1 hour. After adding 30 μg of tRNA (Gibco BRL, Gaithersburg, MD, U.S.A.), the sample was extracted with 400 μl of phenol:chloroform:isoamyl alcohol (Gibco BRL). The aqueous phase was removed and extracted twice with 400 μl of chloroform; then 65 μl of 2N sodium acetate, 3 μg tRNA and 455 μl of isopropanol was added and the samples incubated overnight at −20°C to precipitate RNA. The precipitate was washed with 70% alcohol, air-dried and redissolved in 10 μl of diethyl pyrocarbonate-treated water. After resuspension in 10 μl nuclease-free water, 2 mM primer 2·2 was added and warmed to 70°C for 5 min. The RNA was added to a reverse transcription reaction containing 10 U avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, U.S.A.), 40 U rRNasin (Promega), 200 μM dNTP's, in reverse transcription buffer (Promega). Reverse transcription was performed for 2 hours at 42°C.

Ten μl aliquots of cDNA were transferred to a PCR mixture containing 2·5 U Taq DNA polymerase (Promega), 0·5 mM primer 2·1, 0·5 mM primer 2·2, 200 μM dNTP, and 1 mM $MgCl_2$, in PCR buffer (Promega). Amplification was performed for 40 cycles of 95°C, 1 min; 55°C, 1 min; and 75°C, 2 min. Amplified DNA was analysed on a 2% agarose gel and stained with ethidium bromide. Plasmid pBV-KPB was used as a positive control DNA. The PCR titer for BVDV was defined as the highest dilution of sample which produced a positive reaction in duplicate.

Chimpanzee studies

All chimpanzees used in the study were colony born and bred at the White Sands Research Center, Alamogordo, New Mexico, which is operated in compliance with the Public Health Service Policy on Humane Animal Care and Use and the United States Department of Agriculture's Animal Welfare Act. At the start of the study all serological markers for hepatitis viruses A, B and C were negative for all three chimpanzees.

Human plasmas, collected by plasmapheresis and found to be non-reactive for hepatitis B surface antigen, antibody to human immunodeficiency virus, antibody to HCV and with alanine aminotransferase (ALT) levels less than twice the upper limit of normal values, were pooled. This plasma pool was later found to transmit HCV to chimpanzees.[14] IGIV was fractionated from this plasma as described above. The IGIV in its final liquid formulation, 5% protein, at pH 4·25, was incubated at 21°C for 21 days. It was inoculated into chimpanzee no. 144 at a dose of 1 g/kg body weight. A total of 474 ml of IGIV was infused over a period of 121 min. During this time the animal's temperature and blood pressure were monitored and remained within normal limits. The animal was held for observation for 1 year.

To demonstrate the virucidal effect of the pH 4·25 incubation step on HCV, the same lot of IGIV described above was spiked with HCV to a final concentration of 10^3 chimpanzee infectious doses (CID)/ml. This material was incubated at 21°C for 21 days and then infused into two chimpanzees, no. 191 and no.192, for detection of residual infectious virus. The inoculum per chimpanzee was 10 ml containing the equivalent of 10^4 CID of HCV. Following the infusion, the chimpanzees were held for observation for 36 weeks. At this time both animals were challenged with 10^3 CID of HCV to demonstrate their susceptibility to HCV infection and the infectivity of the HCV used to spike the IGIV. The animals were then held for observation of HCV infection.

HCV detection by nested PCR

Hepatitis C viral RNA from chimpanzee serum samples was detected using a nested PCR as described by Yei.[15] RNA was extracted from the samples by the procedure used for BVDV extraction, except that precipitated nucleic acid was resuspended in 50 µl of diethyl pyrocarbonate-treated water containing 16 U of recombinant RNasin. The four primer sequences were derived from the 5'-noncoding region of the HCV genome. Primer no. 1, 5'-ACTCC ACCAT AGATC ACTCC (nucleotide 7–26) and primer no. 2, 5'-GGTGC ACGGT CTACG AGACCT (nucleotide 304–324, reverse polarity) were used for reverse transcription and the first cDNA amplification. Primer no. 3, 5'-ACTCC CCTGT GAGGA ACTACT (nucleotide 22–42), and primer no 4, 5'-AACAC TACTC GGCTA GCAG (nucleotide 229–248 reverse polarity) were the inner primers and were used in the second amplification. All experiments included an HCV-positive serum control, a negative control, and an RNA control consisting of an RNA transcript of a plasmid derived from the first 400 nucleotides of the viral genome. The identity of the amplified product was confirmed by restriction enzyme digest with Nco 1 (Promega), which cleaves at position 71, and Xma 1 (Promega), which cleaves at position 116. For Nco 1 digestion, 30 µl of amplified product was incubated with 6 U of enzyme in 6 mM Tris-HCl, 150 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, pH 7·9 for 1·5 h at 37°C. For Xma 1 digestion, 30 µl of amplified product was incubated with 2·5 U of enzyme in 6 mM Tris-HCl, 50 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, pH 7·5 for 1·5 h at 37°C. Amplified product and fragments were electrophoresed on a 2% agarose gel and visualized under ultraviolet illumination after ethidium bromide staining.

Results

BVDV distribution during cold ethanol fractionation

BVDV was used as a surrogate for HCV to study the distribution of a pestivirus during the manufacturing process of IGIV. The fractionation scheme of cryoprecipitate-free plasma to IGIV is shown in Fig. 1. As shown in Table 1 the starting cryoprecipitate-free plasma contained $9·0 \times 10^6$ pfu of added BVDV.

Table 1. BVDV distribution during ethanol fractionation

Fraction	Volume (ml)	pfu/ml	total pfu
Cryo-free plasma	100·0	$9·00 \times 10^4$	$9·0 \times 10^6$
Fraction I	32·0	$>2·5 \times 10^4$	$>7·9 \times 10^5$
Supernatant I	103·0	$7·75 \times 10^4$	$7·9 \times 10^5$
Fraction II + III	66·2	$9·25 \times 10^4$	$6·1 \times 10^5$
Supernatant II + III	108·0	$1·00 \times 10^3$	$1·1 \times 10^5$
Fraction II + IIIw	84·6	$>2·5 \times 10^4$	$>2·1 \times 10^6$
Supernatant II + IIIw	79·0	$7·50 \times 10^1$	$5·9 \times 10^3$
Fraction III	100·0	$2·32 \times 10^4$	$2·3 \times 10^5$
Supernatant III	94·0	$<2·0 \times 10^1$	$<1·9 \times 10^9$

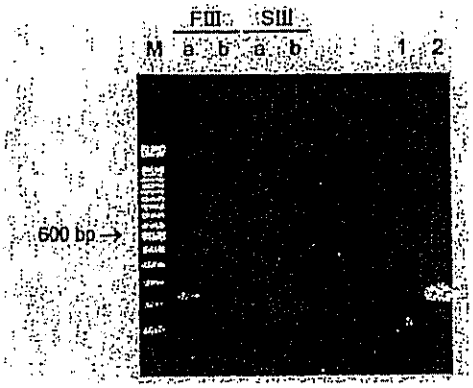

Figure 2. Detection of BVDV RNA by PCR during fractionation of cryoprecipitate-free plasma to IGIV. Amplified products were applied to agarose gels. (M) 100 base pair DNA ladder; (FIII) fraction III; (SIII) supernate III; (a) undiluted sample; (b) 10× diluted sample; (1) IGIV; (2) plasmid pBV-KPB.

Following addition of ethanol to 8% at pH 7·5, −2° to −3°C, the precipitate and supernate, fraction I and supernate I respectively, were separated by centrifugation. Both fraction I and supernate I contained large amounts of BVDV. For precipitation of fraction II + III, the alcohol content of supernate I was raised to 21%, the pH adjusted to 6·75 and the temperature lowered to between −4·5° and −5°C. The resultant precipitate contained $6·1 \times 10^6$ pfu of BVDV while the supernate contained $1·1 \times 10^5$ pfu. Resuspension of fraction II + III in water and addition of ethanol to 20% at pH 6·61, −5° to −6·5°C resulted in fraction II + IIIw and supernate II + IIIw containing $\geq 2·1 \times 10^6$ and $5·9 \times 10^3$ pfu of virus respectively. Resuspension of fraction II + IIIw in water and the addition of ethanol to 17% at pH 5·4, −5·5°C to −6·5°C resulted in fraction III containing $2·3 \times 10^6$ pfu of virus and no virus detectable in supernate III.

Fraction II + III, fraction III, and supernate III were tested for the presence of viral RNA by PCR. While fraction II + III and fraction III were positive for viral RNA, supernate III contained no viral RNA (Fig. 2). The sensitivity of PCR for BVDV was a minimum of 100 pfu.

Inactivation of BVDV in IGIV

The effect of pH and time on viral inactivation in IGIV solutions was measured. As shown in Fig. 3a, BVDV incubated in IGIV at different pHs for 21 days at 21°C, showed decreasing titers with decreasing pH. When incubated in IGIV at pH 4·25, 21°C, BVDV showed a 10 000-fold decrease in titer over 21 days. A 10-fold decrease in PCR titer was found after 7 days, but thereafter remained constant (Fig. 3b).

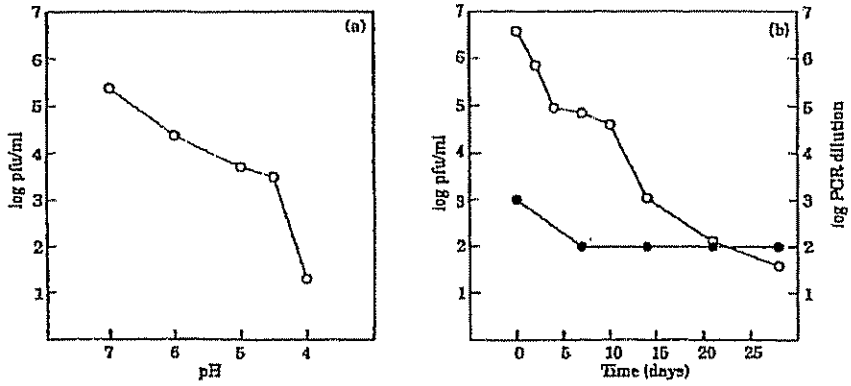

Figure 3. Inactivation of BVDV in IGIV. (a) BVDV was assayed for infectivity after incubation in IGIV for 21 days at 21°C at the pH indicated. (b) Incubation of BVDV in IGIV pH 4·25 at 21°C. Samples were assayed for residual infectivity (O) and for PCR titre (●) after different incubation times.

Inactivation of HCV in IGIV

The results of weekly monitoring over a 1-year period of chimpanzee no. 144 inoculated with IGIV fractionated from infectious plasma for signs of HCV infection are shown in Fig. 4. The ALT values remained within normal limits throughout this period. There was no indication of HCV infection either by detection of virus in the serum by PCR or detection of anti-HCV by the first generation ELISA from Abbott Laboratories (Abbott Park, IL, U.S.A.). When it became available, the second generation test was used to retest all samples; the results, however, between the two tests were not different.

The results of tests on the weekly serum samples collected from chimpanzees no. 191 and no. 192, inoculated with the HCV-spiked IGIV, are shown in Fig. 5. Throughout the 36-week observation period, ALT levels remained within normal limits; tests for viremia by PCR were negative, and anti-HCV assays by both first and second generation tests were also negative.

At the end of the 36-week observation period, both animals were challenged with 10^4 CID of the HCV used as spike for the IGIV. As shown in Fig. 5, both chimpanzees became viremic after challenge. The sera became positive for virus by PCR at two weeks, followed by a rise in ALT levels. Chimpanzee no. 191 had elevated ALT values from the ninth to 10th week after challenge and chimpanzee no. 192 showed a rise in ALT above normal on the 10th to 11th week after challenge. Both chimpanzees seroconverted to anti-HCV positivity on the 11th week post-challenge; however, chimpanzee no. 191 on subsequent weeks was antibody negative even when retested by second generation tests.

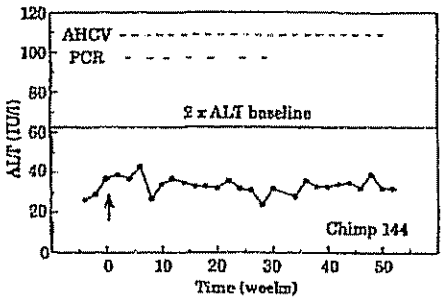

Figure 4. Monitoring of chimpanzee no. 144 for indications of HCV infection following infusion of IGIV. Arrow indicates day of infusion. Data lines above the figure indicate results of PCR and antibody testing (AHCV).

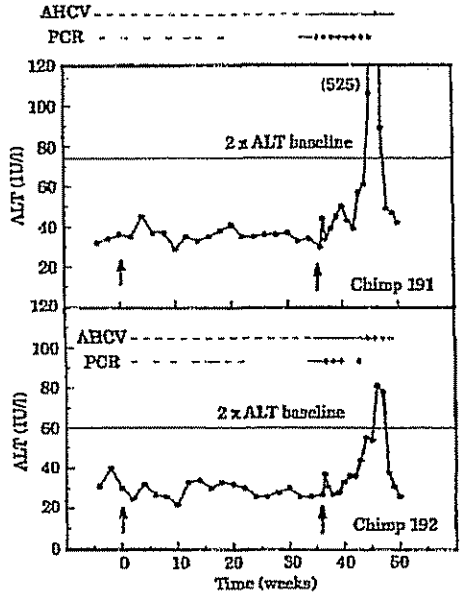

Figure 5. Monitoring of chimpanzee no. 191 and no. 192 for indications of HCV infection following infusion of IGIV spiked with HCV and inactivated by pH 4.25. Thin arrow indicates day of infusion and thicker arrow indicates day of challenge with HCV. Data lines above the figure indicate results of PCR and antibody testing (AHCV).

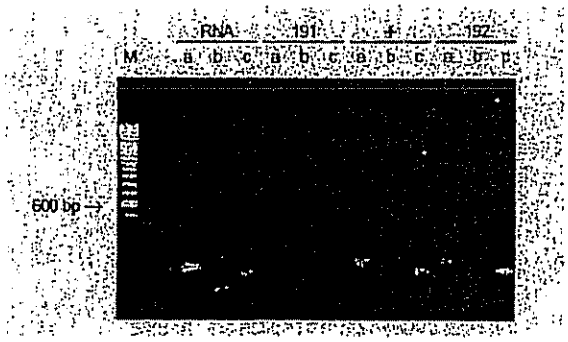

Figure 6. Detection of HCV RNA by PCR in 2-week post-challenge chimpanzee sera. Amplified products were applied to agarose gels. (M) 100 base pair DNA ladder; (RNA) HCV RNA positive control; (191) sera from chimpanzee 191; (+) HCV positive plasma control; (192) sera from chimpanzee 192. (a) amplified product; (b) Xma 1 digestion; (c) Nco 1 digestion.

The PCR results of the 2-week post-challenge sera are shown in Fig. 6. Included here are the positive controls, an HCV positive plasma and an RNA transcript representing the first 400 nucleotides of the HCV genome. Specificity of the PCR product is demonstrated by the identical molecular weight products from the serum samples and positive controls before and after treatment with Xma 1 and Nco 1 restriction enzymes.

Discussion

In our examination of the clearance of BVDV through the IGIV manufacturing process, the data show that with each successive fractionation step, there was a progressive decrease of virus in the resultant supernate such that the stage from which IGIV is directly obtained, supernate III, was devoid of virus. The bulk of the virus was carried in the precipitated fractions. The percent ethanol and acidic conditions used for each fractionation step have little virucidal effect, perhaps due to the low temperatures at which the reactions were carried out In the absence of inactivation, elimination of virus through this manufacturing process appears to be due entirely to partitioning of the virus into the precipitated fractions, as viral RNA not associated with infectivity cannot be detected by PCR beyond the final precipitate. These results are qualitatively similar to results reported by Yei[15] on the partitioning of HCV through the same process. The quantitative differences seen were in the supernatant fractions; less HCV was detected in supernate II + III and none in succeeding supernates II + IIIw and III. These differences may be due to a more efficient partitioning of HCV as immune complexes into the precipitated fractions since the work was carried out on a pool of anti-HCV reactive plasmas whereas with the present study, although not tested, one would not expect BVDV antibodies in human plasmas. However, now that anti-HCV screening of human plasmas used for manufacture of therapeutic products has been instituted, HCV could be present in the plasma pools as the result of accepting plasmas from infected donors who have not yet seroconverted. In these instances the virus partitioning through the manufacturing process should be more similar to that described for BVDV.

In addition to the use of the Cohn-Oncley fractionation process, the manufacture of IGIV includes an incubation of the liquid product in its final container at 21°C to 27°C for 21 to 24 days. This incubation step serves two purposes: examination of the containers at the end of this period allows a 100% inspection of them for microbial contamination; and utilization of the acidic medium for virus inactivation, the effectiveness of which against retroviruses has been demonstrated.[3,16] Using BVDV as a surrogate pestivirus for HCV, the virucidal effect of the pH of IGIV solutions was shown to increase with increasing acidity of the medium. At a pH of 4·25, the infectivity of BVDV in IGIV was decreased over 10 000-fold during 21 days at 21°C.

Definitive proof that the IGIV produced by the process described is safe from HCV was provided by the data from inoculation of IGIV into chimpanzees. Despite the presence of infectious HCV in this particular pool of anti-HCV negative plasmas,[14] the IGIV produced from it was shown to be noninfectious after infusion of 474 ml into a chimpanzee. The effectiveness of the pH 4·25 inactivation step was demonstrated by inoculation of the above lot of IGIV with 10^3 CID of HCV per ml and incubated under the prescribed conditions, into two chimpanzees. These animals showed no signs of HCV infection after 36 weeks. At the end of this period, the animals challenged with HCV became viremic as evidenced by detection of viral RNA in serum, by elevated ALT levels, and by anti-HCV seroconversion thereby demonstrating their susceptibility to HCV and the infectivity of the HCV used to spike the IGIV.

The capacity of the IGIV manufacturing process to eliminate HCV infectivity may be estimated according to the European Communities Note for Guidance entitled 'Validation of virus removal and inactivation procedures (111/8115/89-EN)'. Because each liter of plasma yields 60 ml of 5% IGIV, the 474 ml of IGIV given to chimpanzee no. 144 was produced from 7900 ml of pooled plasma containing $1·6 \times 10^3$ CID/ml of HCV.[15] The potential infectious load of HCV given to the chimpanzee was $1·3 \times 10^7$ CID [(7900 ml) ($1·6 \times 10^3$ CID/ml)]. Because the chimpanzee did not become infected, the final titer is <1 CID. Therefore, the production process, including fractionation and low pH incubation, had a calculated inactivation factor of $\geq 1·3 \times 10^7$ [($1·3 \times 10^7$ CID)/(<1 CID)] or a log inactivation of ≥7·1. When the low pH incubation step was separately evaluated by inoculation of two chimpanzees with IGIV spiked and incubated with HCV, 10 ml of 1×10^7 CID/ml or 1×10^4 CID was infused into each animal. Because the chimpanzees were not infected, the final titer was <1 CID. Therefore, the low pH incubation step alone had an inactivation factor of $>1 \times 10^4$ or a log inactivation of >4. These values should be considered conservative estimates because the inactivation

and clearance of HCV through the fractionation steps and the low pH incubation were not tested to their limits.

The clinical experience with IGIV confirms the safety of the product under these conditions of manufacture. In a clinical study of the safety of intravenous globulin by Rousell,[17,18] 1777 50 ml vials or 88,500 ml of one lot of IGIV (Dr R. Rousell, personal communication) prepared from plasmas not screened for anti-HCV were administered over an extended period of time to primary immunodeficiency patients. Under subsequent close surveillance none of these patients developed any indication of HCV infection.

In conclusion, with consideration of the following points, that the viral load of plasma pooled after screening for anti-HCV was reduced to less than 10^4 CID/ml[15] and that the chimpanzee data show at least a $10^{7.1}$ fold reduction of HCV, the manufacturing process including the low pH virucidal step should result in an IGIV which is free from HCV transmission.

Acknowledgements

The authors gratefully acknowledge the assistance of Mr Donald L. Tankersley and Dr Soon Yei, Center for Biologics Evaluation and Research, Bethesda, Maryland, U.S.A. for issues related to the HCV PCR assay. Dr Robert Eason and Ms Deborah Dockum, White Sands Research Center, Alamogordo, New Mexico are to be thanked for overseeing the chimpanzee studies at the White Sands Research Center.

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EXHIBIT 11

United States Patent [19]

Mitra et al.

[11] Patent Number: 4,762,714

[45] Date of Patent: Aug. 9, 1988

[54] PREPARATION OF RETROVIRUS-FREE IMMUNOGLOBULINS

[75] Inventors: Gautam Mitra, Kensington; Milton M. Mozen, Berkeley, both of Calif.

[73] Assignee: Miles Laboratories, Inc., Elkhart, Ind.

[21] Appl. No.: 849,612

[22] Filed: Apr. 8, 1986

[51] Int. Cl.[4] A61K 35/14; A61K 39/395; C07K 15/06; C12N 9/00

[52] U.S. Cl. 424/101; 424/85; 424/89; 435/236; 530/387; 514/2

[58] Field of Search 424/101, 85, 89; 435/236; 530/387; 514/2

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2,897,123	7/1959	Singher	424/101
4,396,608	8/1983	Tenold	424/101
4,440,679	4/1984	Fernandes et al	424/101
4,640,834	2/1987	Eibl et al	424/101

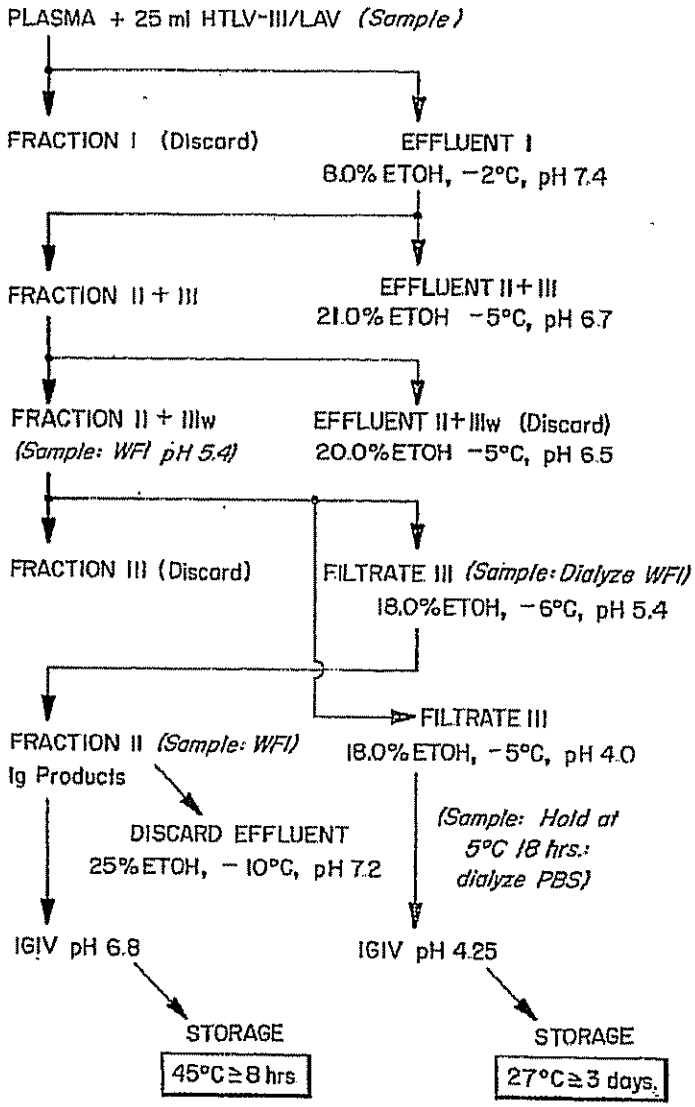
Primary Examiner—Alan Siegel
Assistant Examiner—Jacqueline M. Stone
Attorney, Agent, or Firm—James A. Giblin

[57] ABSTRACT

Immune serum globulins (ISG) can be made substantially free of infectious retroviruses by preparing the ISG from human plasma using a cold ethanol plasma fractionation process at a pH equal to or less than 5.4 and then storing the ISG at either of two specified storage conditions: (1) at a pH equal to or less than about 4.25 at a temperature of about 27° C. for at least 3 days, or (2) at a pH equal to or less than about 6.8 at a temperature of about 45° C. for at least about 8 hours.

2 Claims, 1 Drawing Sheet

HTLV—III/LAV Plasma Fractionation



PREPARATION OF RETROVIRUS-FREE IMMUNOGLOBULINSpg,10

BACKGROUND OF THE INVENTION

1. Field

This disclosure is concerned generally with the inactivation of retroviruses in immune serum globulin (ISG) and specifically with the inactivation of such retroviruses as the LAV strain of an AIDS virus in ISG intended for intravenous (IV) administration

2. Prior Art

Therapeutic and prophylactic ISG preparations are well known and have been available for many years. ISG is presently obtained in commercial quantities using variations of a blood plasma fractionation technique developed by Cohn et al in the 1940's. Although ISG has been administered intramuscularly (IM) and more recently intravenously (IV), the latter route of administration provides numerous advantages and has gained acceptance as the preferred route of administration.

Initial attempts to render an ISG safe and effective for IV administration (IVIG) focused on eliminating its anticomplement activity. In one approach, for example, this involved chemically modifying the ISG (see U.S. Pat. No. 3,903,262 to Pappenhagen et al). More recently, the ISG has been made suitable for IV administration through careful pH and ionic strength control (see U.S. Pat. No. 4,396,608 and U.S. Pat. No. 4,499,073 both to Tenold). It is also known that IVIG preparations can be stabilized with carbohydrates such as maltose (see U.S. Pat. No. 4,186,192 to Fernandes et al). ISG preparations can be further purified using a variety of techniques (see, for example, U.S. Pat. No. 4,272,521 to Zuffi). Various ISG preparations having a relatively high titer to a given antigen are also well known (e.g. tetanus, hepatitis, Rho factor, etc.).

Although ISG products (both IMIG and IVIG) have been considered generally safe, there has been a growing need to assure patients that ISG products do not transmit active viruses such as those associated with hepatitis or, more recently, retroviruses such as that associated with Acquired Immune Deficiency Syndrome (AIDS). The present disclosure is based on work done to address such needs.

Antibodies to a retrovirus associated with the AIDS have been detected in human hepatitis B immunoglobulin (HBIG) (see Tedder, R. S. et al, Safety of immunoglobulin preparation containing anti-HTLV-III, Lancet 1985;1:815) as well as in other commercial lots of immunoglobulins (see Gocke, D. J. et al, HTLV-III antibody in commercial immunoglobulin, Lancet 1986;1:37–8). This observation raised the possibility that immunoglobulin products transmit infectious virus. This concern was heightened by recent reports of non A, non B (NANB) hepatitis in immunodeficient patients who had received infusions of intravenous immunoglobulins prepared from Cohn fraction II (see Webster, A. D. B. et al, Non-A, non-B hepatitis after intravenous gammaglobulin, Lancet 1986;i:322, and Ochs, H. D. et al, Non-A, non-B hepatitis after intravenous gammaglobulin, Lancet 1986;1:322–23).

Based on the above findings, we decided to determine the ability of retroviruses to withstand the various procedures employed in immunoglobulin preparations as well as other procedures. For these experiments, two prototype retroviruses were used: the mouse xenotropic type C retrovirus and the LAV strain of the AIDS retrovirus. Surprisingly, we found that the model retroviruses could be inactivated in ISG prepared by a known fractionation processing technique if that technique is followed by storage at controlled conditions of pH, temperature and time. Details of our method are described below

SUMMARY OF THE INVENTION

We have found that ISG preparations can be made substantially free of retrovirus such as a LAV strain associated with AIDS by preparing the ISG from pooled plasma using a known processing technique (i.e. Cohn-Oncley cold ethanol process, using at least about 18% ethanol v/v at pH 5.4), followed by storage of the ISG at a pH of less than 5.4, a temperature of at least about 27° C., or at a pH of 6.8 at a temperature of at least about 45° C. for periods sufficient to assure retrovirus inactivation. In preferred embodiments, our ISG preparation is stabilized with a carbohydrate (e.g. maltose) and in a 5% wt./vol. liquid (aqueous) form. It is intended for IV use and is made substantially free (less than 10 infectious virus particles) of the LAV strain of retrovirus associated with AIDS by processing pooled human plasma using the Cohn-Oncley cold ethanol process (about 18% ethanol, pH≅5.4) to obtain ISG followed by storage of the ISG at a pH of about 4.25 for at least about 21 days at a temperature about 27° C. In another embodiment, the ISG may be stored at pH 6.8 for about 45° C., for at least 8 hours to assure the retrovirus inactivation.

BRIEF DESCRIPTION OF THE FIGURE

The FIGURE illustrates a flow chart of the steps used in our Cohn-Oncley cold ethanol fractionation of human plasma, including the novel storage conditions disclosed herein.

SPECIFIC EMBODIMENTS

Materials and Methods

The mouse xenotropic type C retrovirus recovered from a New Zealand Black mouse kidney was grown to high titer in mink lung cells (Varnier, O. E. et al, Murine xenotropic type C viruses V. Biological and structural differences among three cloned retroviruses isolated from kidney cells from one NZB mouse, Virology 1984;132:79–94). Detection was based on a focus assay in mink S+L-cells in which each infectious particle scores as an area of cell transformation (Peebles, P. T., An in vitro focus induction assay for xenotropic murine leukemia virus, feline leukemia virus C, and the feline Primate viruses RI-114/CCC/M-7, Virology 1975;67:288–91). Virus titer was also determined by the induction in cells of the viral core structural protein (page 30) measured by immunofluorescence (see Levy, J. A., Xenotropic type C viruses, Current Topics Microbiol. Immunol. 1978;79:111–212). The use of these assays for detection of mouse C virus in spiking experiments with plasma fractions has previously been described by us (see Levy, J. A. et al, Recovery and inactivation of infectious retroviruses added to factor VIII concentrates, Lancet 1984;ii:722–723 and Levy, J. A. et al, Inactivation by wet and dry heat of AIDS-associated retroviruses during factor VIII purification from plasma, Lancet 1985;i:1456–1457).

LAV was cultured and obtained from the Centers for Disease Control (CDC) in Atlanta, Ga. Its detection

was based on a sandwich enzyme-linked immunoassay (ELISA) previously described (see McDougal, J. S et al, Immunoassay for the detection and quantitation of infectious human retrovirus, lymphadenopathy-associated virus [LAV], J. Immunol. Methods 1985;76:171–183).

Human plasma samples were spiked with retroviral preparations and fractionated according to classical Cohn-Oncley cold ethanol procedures (see Cohn, E. J. et al, Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of protein and lipoprotein components of biological tissues and fluids, J. Am. Chem. Soc. 1946;68:459–75 and Oncley, J. L. et al, The separation of the antibodies, isoagglutinins, prothrombin, plasminogen, and beta-1-lipoprotein into subfractions of human plasma, J. Am. Chem. Soc 1949;71-541-50). The fractionation was accomplished through selective precipitations in the cold at various ethanol concentrations and pH values: fraction I at 8% ethanol, −2° C., pH 7.4; fraction II-+III at 21% ethanol, −5° C., pH 6.7; fraction II+IIIw at 20% ethanol, −5° C., pH 6.5; fraction III at 18% ethanol, −6° C., pH 5.4; and fraction II collected at 25% ethanol −10° C., pH 7.2. Residual retroviral levels were determined across the fractionation steps. The pH (range 5.4–4.0) and temperature (range −5° C. to 22° C.) effects on virus infectivity in the presence of ethanol (approximately 18%) were determined with filtrate III. Final container liquid immunoglobulin preparations, in the absence of ethanol, were incubated with retrovirus concentrates at 27° C and 45° C.; virus infectivity was determined at different time periods.

From plasma to fraction II+IIIw, no more than a 10-fold reduction of virus titer was observed. Preparation of filtrate III from fraction II+IIIw resulted in an approximately 10,000-fold reduction of the mouse type C retrovirus and 10-fold reduction in LAV. Due to dilution, ethanol concentration decreased from 20% v/v to 18% v/v across this fractionation step and the pH was reduced from 6.50 to 5.40. Fraction II precipitation from filtrate III resulted in >1,000-fold reduction in titer of both the infectious mouse and human retroviruses. During this fractionation step, the pH was raised to 7.25 and the ethanol concentration increased to 25%. The 1,000-fold loss of virus infectivity primarily results from virus inactivation (not fractionation) since after extensive dialysis, no infectious virus was measurable in the supernatant corresponding to fraction II (data not shown).

In studying more precisely the effect of pH and temperature on retrovirus inactivation with 18% ethanol, we mixed a quantity of the mouse retrovirus with filtrate III. See Table 2.

TABLE 2

Effect of pH and Temperature on Mouse Type C Retrovirus added to Filtrate III (18% Ethanol)

	Temperature −5° C.				Temperature 22° C.	
	(a) pH 5.4	(b) pH 4.7	(c) pH 4.0		(d) pH 5.4	(e) pH 4.0
Sample	(Total IP)	(Total IP)	(Total IP)	Sample	(Total IP)	(Total IP)
Virus alone	7.9×10^6	7.9×10^6	7.9×10^6	Virus alone	4.0×10^8	5.0×10^7
Virus + filtrate III	2.9×10^6	3.4×10^5	6.5×10^5	Virus + filtrate III	2.2×10^7	5.5×10^5
2 hours	3.4×10^5	6.6×10^6	2.0×10^5	3 hours	2.2×10^8	Non-detectable
4 hours	6.7×10^5	6.7×10^5	2.6×10^5			
6 hours	7.8×10^5	6.6×10^5	4.1×10^5			

Total infectious particles were detected as described under Materials and Methods. Detection limit approximately $10^{-1.3}$ IP/ml. Two ml of mouse type C virus concentrate was added to 20 ml of effluent III for each of (a), (b), and (c). Ten ml and 5 ml of mouse type C virus were added to 100 ml and 50 ml of effluent III, respectively for (d) and (e).

Results

Infectivity of both the mouse C and AIDS retrovirus was not affected by the addition of these viruses to human plasma at ≦5° C. See Table 1.

TABLE 1

Effect of Immunoglobulin fractionation procedures on infectious retrovirus added to plasma

Stage	Mouse Type C (Total IP)	AIDS Virus LAV (Total ID_{50})
Virus alone	2.0×10^6	2.3×10^5
Virus + plasma (5° C.)	2.3×10^8	4.4×10^5
II + IIIw	3.8×10^7	4.8×10^4
Filtrate III	1.6×10^3	1.7×10^3
Fraction II	Non-detectable	Non-detectable

Twenty ml of virus concentrate was added to 200 ml of plasma for the fractionation studies described. The fractionation methods and viral assays are described in the text. Total IP = total infectious particles. Total ID_{50} = ID_{50}/reaction(s) of dilution at which 50% of the cultures are positive. + volume

At −5° C., no significant virucidal effect was seen in the pH range of 5.4–4.0 for up to 6 hours (2*a*, *b*, *c*). At 22° C. (ambient), however, at pH 4.0>100,000 infectious mouse retrovirus particles were inactivated by 3 hours (2*e*). In contrast, at pH 5.4 under similar conditions, no significant virucidal effect was seen (2*d*). Similarly, 1.7×10^3 total ID_{50} of LAV that was in a filtrate III solution at pH 4.0 and held at +5° C. for 18 hours, was reduced in titer to non-detectable level (data not shown). It therefore appears that the presence of 18% ethanol in plasma fractions at pH 5.4 is not markedly virucidal for these viruses in the temperature range of −5° C. to 22° C. Only when the pH is lowered (pH 4.0) concomitant with a raise in temperature (≧5° C.), significant virus inactivation observed. For LAV, the following conditions were sufficient for a 1,000-fold reduction in infectious virus: ethanol 18%, pH 4.0, temperature +5° C., time 18 hours (data not shown). For the mouse type C retrovirus, >10,000-fold reduction was measured under similar treatment conditions. To determine the effect on AIDS virus of pH and temperature of the final product, final container liquid immunoglobulin preparations (protein concentration 5% w/v) were incubated with LAV (Table 3). At 27° C., between 10^3–10^4 of total ID_{50} were inactivated by 3 days for the immunoglobulin preparations of both pH 6.8 and pH 4.25. At 45° C., >10,000 infectious particles were inactivated within 8 hours with the pH 6.8 immunoglobulin preparation. The pH 4.25 immunoglobulin preparation was not tested at 45° C.

Discussion

These experiments were conducted to evaluate the effect on infectious retroviruses of procedures used for immunoglobulin fractionation. The data are important in evaluating the possible risk of AIDS virus contamination of some Ig preparations. The mouse type C retrovirus was used as well as the LAV strain of AIDS virus, because the former can be grown to very high titer and therefore the effect of various procedures can be better evaluated. In addition, a focus assay for the mouse virus allows more precise quantitation.

Unlike the reported complement-mediated lysis of many retroviruses in human serum at 37° C (see Welsh, R. M. et al, Human serum lysis RNA tumor viruses, Nature 1975;257:612–14), the AIDS virus in the cold (0°–5° C.) is not affected by this mechanism (see Banapour, B. et al, The AIDS-associated retrovirus is not sensitive to lysis or inactivation by human serum, Virology [in press] 1986). The reported virucidal effects of ethanol for LAV have been at ambient temperature (see Spire, B. et al, Inactivation of lymphadenopathy associated virus by chemical disinfectants, Lancet 1984;ii:8-99–901 and Martin, L. S. et al, Disinfection and inactivation of human T lymphotropic virus type III/ lymphadenopathy associated virus, J. Infec. Dis. 1985;152:400403), whereas the data reported here show that these virus inactivating effects are diminished in the presence of plasma at low temperatures (<45° C.). Enhanced inactivation at low pH is demonstrated which again is strongly dependent on temperature. This observation agrees with an earlier report (see Martin, L. S. et al, Disinfection and inactivation of human T lymphotropic virus type III/lymphadenopathy associated virus, J. Infec. Dis. 1985; 152:400–403) indicating increased inactivation of LAV inoculum at pH extremes.

Filtrate III with 18% ethanol at pH 5.4 and at a temperature of −5° C. was not significantly virucidal for retroviruses for extended periods of time. Hence, the 100,000-fold reduction of the mouse type C virus and a 100-fold reduction of LAV from plasma to filtrate III is probably primarily due to fractionation under the processing condition (ethanol range 0–20% v/v, pH range 7.4–5.4) employed at −5° C. The reduction difference between the mouse and the human virus reflects either a greater resistance of the AIDS virus to the processing conditions or a less quantitative assay for this virus. As noted above, the mouse virus can be grown up to high titers and its assay is very reproducible. Its usefulness for fractionation/inactivation studies has been previously reported by us (see Levy, J. A. et al, Recovery and inactivation of infectious retroviruses added to factor VIII concentrates, Lancet 1984;ii:722–723 and Levy, J. A. et al, Inactivation by wet and dry heat of AIDS-associated retroviruses during factor VIII purification from plasma, Lancet 1985;i:1456–1457).

Ethanol concentration is increased to 25% v/v at pH 7.20 for the fraction II precipitation which results in a more than 1,000-fold inactivation of the mouse type C virus and LAV. Since the corresponding effluent was free of infectious virus, true inactivation at the 25% ethanol concentration is most likely involved. A recent report (see Piszkiewicz, D. et al, Inactivation of HTLV-III/LAV during Plasma fractionation, Lancet 1985;ii:1188–89) had shown inactivation of $10^{4.5}$ ID_{50} of the AIDS retrovirus during the precipitation of I+II+III (ethanol 20% v/v, pH 6.9, temperature −5° C.) under conditions in which fraction II+III is precipitated together with fraction I. Our results which isolate these fractions separately do not show such complete LAV inactivation under similar conditions (Table 1). In our study, the samples were extensively dialyzed in PBS prior to ID_{50} assay. In the other report, a 1:10 dilution to a resultant residual ethanol concentration of 2% v/v was used in the assay. Furthermore, it is not possible from the other report to distinguish whether the virus titer was being determined in the precipitate or the supernatant following I+II+III precipitation; hence, a meaningful comparison between the two studies is difficult to make.

Greater than a 1,000-fold drop in AIDS virus infectivity did result after its incubation with purified liquid immunoglobulin preparations at 27° C. for 3 days; pH of the purified immunoglobulin preparations did not seem to have an appreciable effect. A higher incubation temperature (45° C.) demonstrated comparable titer reduction within 8 hours. A "worse case" estimate of 2,000 ID/ml of AIDS virus in large plasma pools has been reported (see Petricciani, J. C. et al, Case for concluding that heat-treated, licensed antihemophilic factor is free from HTLV-III, Lancet 1985;ii:890–891). The yield of IgG could be as low as 50% of the amount present in plasma together with IgG concentration increase from approximately 1 gm/100 ml in plasma to 5 gm/100 ml in purified product. If the AIDS virus was concentrated without loss of infectivity along with IgG purification, the purified IgG would contain 2,000 ID/ml×10 (2×10^4 ID/ml) Immunoglobulin purification processes must therefore be able to fractionate/inactivate 2×10^4 ID/ml of AIDS virus.

No single step in the Cohn cold ethanol process can completely inactivate retroviruses. The effects of fractionation and inactivation taken together through the fractionation cascade could be quite large. LAV recovery from plasma to fraction II is reduced by at least 100,000-fold; pH adjustment to 4.0 at filtrate III (at +5° C.) is as effective for viral inactivation as precipitation of fraction II in the presence of 25% ethanol. An extra margin of safety is provided when the final preparation in liquid form is incubated at 27° C., since these experiments demonstrated that in liquid immunoglobulin preparations, a 1,000–10,000-fold reduction of LAV occurred within 3 days under these conditions. Prince et al, Effect of Cohn fractionation conditions on infectivity of the AIDS virus, N. Eng. J. Med 1986; 314:386–87, have suggested that the long storage of liquid immune serum globulin preparations may contribute to their safety. The studies presented here experimentally validate that AIDS virus are indeed inactivated during liquid storage. See Table 3.

TABLE 3

Effect of pH and Temperature on LAV added to Final Container Liquid Immunoglobulin Preparations

	Temperature 27° C.			Temperature 45° C
Sample	pH 6.8 IgG (Total ID_{50})	pH 4.25 IgG (Total ID_{50})	Sample	pH 6.8 IgG (Total ID_{50})
Virus + IgG	1.65×10^4	3.69×10^3	Virus + IgG	1.65×10^4
3 days	Non-detectable	Non-detectable	1 hour	6.27×10^3
12 days	Non-detectable	Non-detectable	4 hours	1.65×10^2
24 days	Non-detectable	Non-detectable	8 hours	Non-detectable
			20 hours	Non

TABLE 3-continued

Effect of pH and Temperature on LAV added to Final Container Liquid Immunoglobulin Preparations

	Temperature 27° C.			Temperature 45° C.
Sample	pH 6.8 IgG (Total ID_{50})	pH 4.25 IgG (Total ID_{50})	Sample	pH 6.8 IgG (Total ID_{50})
				detectable

ID_{50} of LAV as defined in Table 1. ID_{50} detection limit $10^{1.8}$. 1.5 ml of an LAV preparation was added to 15 ml of IgG solution for each of the two parts of the experiment.

The chance for an infectious retrovirus to survive this fractionation as well as storage of the liquid final preparation, is therefore extremely small, if at all.

The fractionation/inactivation and final container incubation results reported here support the available clinical and epidemiological evidence that therapeutic immunoglobulins prepared by Cohn-Oncley cold ethanol process (≧18% v/v ethanol, pH≦5.4 at filtrate III) do not transmit AIDS viruses particularly after storage at a pH of 4.25 at a temperature of 27° C. for about 3 days or at pH 6.8 at temperature of 45° C. for at least 8 hours. The conditions of the Cohn-Oncley process i e., alcohol concentration, pH, temperature, do not in themselves inactivate AIDS virus as recently reported by Prince et al, Effect of Cohn fractionation conditions on infectivity of the AIDS virus, N. Eng. J. Med. 1986;314:386–87. As described, their study was primarily geared towards determining inactivation, and no sequential fractionation was carried out with a virus spike. The present study, in contrast, mimics a true fractionation run and hence portrays a realistic virus carryover estimate involving the sum total of fractionation and inactivation.

It is important to emphasize that variations from classical Cohn approach need to be validated in terms of their virucidal and virus distribution potential since fractionation, ethanol concentration, pH, and temperature all play an important role in virus recovery. It is possible that total log reduction of different viruses could be different and hence it would be difficult to generalize these virus recovery results for other viruses.

However, given the above disclosure, it is thought that variations will occur to those skilled in the art. Accordingly, it is intended that the scope of the invention disclosed should be limited only by the following claims.

We claim:

1. A method of preparing an immune serum globulin substantially free of infectious retroviruses comprising the steps of

(1) preparing an immune serum globulin from a human plasma source using a cold ethanol process at a pH equal to or less than about 5.4 and then

(2) storing the globulin at a pH equal to or less than about 4.25 at a temperature of about 27° C. for at least about 3 days.

2. A method of preparing an immune serum globulin substantially free of infectious retroviruses comprising the steps of

(1) preparing an immune serum globulin from a human plasma source using a cold ethanol process at a pH equal to or less than about 5.4 and then

(2) storing the globulin at a pH equal to or less than about 6.8 at a temperature of about 45° C. for at least about 8 hours.

* * * * *

EXHIBIT 12

Vox Sang. 7: 157–174 (1962)

Intravenous Administration of Human γ-Globulin *

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There are numerous reasons why the administration of human γ-globulin by the intravenous route is to be preferred to the intramuscular application which is widely practised today: the γ-globulin acts more rapidly; there is no loss due to local proteolysis; the greater efficiency of an intravenous administration reduces the cost of treatment; finally, it causes no painful local irritation if large quantities are given [1].

As a routine method, however, intravenous administration of γ-globulin is still not free of risk: untoward reactions may occur with intravenous use of standard γ-globulin; but serious anaphylactoid reactions appear to be rare. Some authors [2] therefore consider the distrust of intravenous injection to be unjustified. Our own experience leads us to think otherwise, and therefore we have studied the phenomenon of intolerance to intravenously applied γ-globulin, with the aim of eliminating its cause.

The following is a summary of our results to date. Therapeutic and prophylactic aspects of intravenous γ-globulin administration, which are basically the same as in intramuscular therapy, are not discussed here.

In order to determine the *frequency of reactions*, 70 individuals were given γ-globulin infusions under the following standard conditions: 10 ml of the commercial, 16% standard γ-globulin (prepared by the Central Laboratory of the Swiss Red Cross Blood Transfusion Service) were added to 100 ml of pyrogen-free 0.9% saline. This yields 110 ml of a 1.45% γ-globulin solution, which is passed through a sterile and pyrogen-free plastic infusion set into the subject's cubital vein, over

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a period of 90 to 120 min. The subject must receive no γ-globulin in any form for a period of at least two weeks before the trial.

Subjects who react under these standard conditions are listed as "sensitive". However, the terms "sensitive" and "non-sensitive" are to be taken relatively because some of the subjects react differently under extreme conditions. Some "sensitive" patients may show no intolerance after very slow infusion of highly dilute γ-globulin solution and conversely, direct injection of the undiluted material to otherwise "nonsensitive" patients may produce symptoms.

A first investigation showed that 21 (or 30 %) of the 70 healthy and sick subjects presented clinical signs of intolerance under standard conditions while 49 (or 70 %) showed no side effects. More valuable information about the risk of reaction emerges if the tested subjects are listed according to kinds of disease. For purely practical reasons the incidence of reactions was studied primarily in patients who required uninterrupted γ-globulin therapy for a γ-globulin and antibody deficiency [3]. The result was unexpected: whereas only 7 (or 13 %) of the subjects without antibody deficiency reacted, 14 (or 92 %) out of 15 patients with an antibody deficiency syndrome showed signs of intolerance. Usually the violence of the reaction correlated with the extent of the defect of antibody synthesis. It appears, however, that the tendency to stronger reactions is not due solely to a reduced serum γ-globulin level; among patients whose hypogammaglobulinemia is due to a loss – either through the kidney (nephrosis) or the gut (protein-loosing enteropathy) – the incidence of "sensitive" recipients

TABLE I

Standard Test

(110 cc 1.45% γ-globulin solution infused in 90–120 min)

Subjects	"Sensitive"	"Non-sensitive"
55 without antibody deficiency	7 (12.7%)	48 (87.3%)
15 with antibody deficiency	14 (93.3%)	1 (6.7%)

was smaller than among patients with an impaired synthesis of γ-globulins.

On the basis of these observations it would be tempting to presume a causal relationship between these subjects' intolerance of intravenously administrated γ-globulin and their inability to synthetize antibodies. No definite proof of the correctness of such an assumption has yet been given. In particular, the chronic infections from which these patients with an antibody deficiency (AD) suffer do not seem to be the only pathogenic factor, since other subjects without AD but with generalized or chronic bacterial infections usually tolerate γ-globulin in the standard test without trouble.

(1) The Clinical Picture of γ-Globulin Intolerance

The symptoms of γ-globulin intolerance are remarkably sterotyped, although the intensity of the manifestations may vary widely: two phases can usually be distinguished in the course of the reaction; the more violent the reaction, the shorter the interval between the two phases.

In the *first phase*, which, under standard conditions, usually develops gradually, some uneasiness, tachycardia with tachypnea and shortage of breath may be observed. Especially characteristic are flushing of the face, feeling of oppression in the chest, and lumbar pain. The blood pressure is constant or slightly elevated.

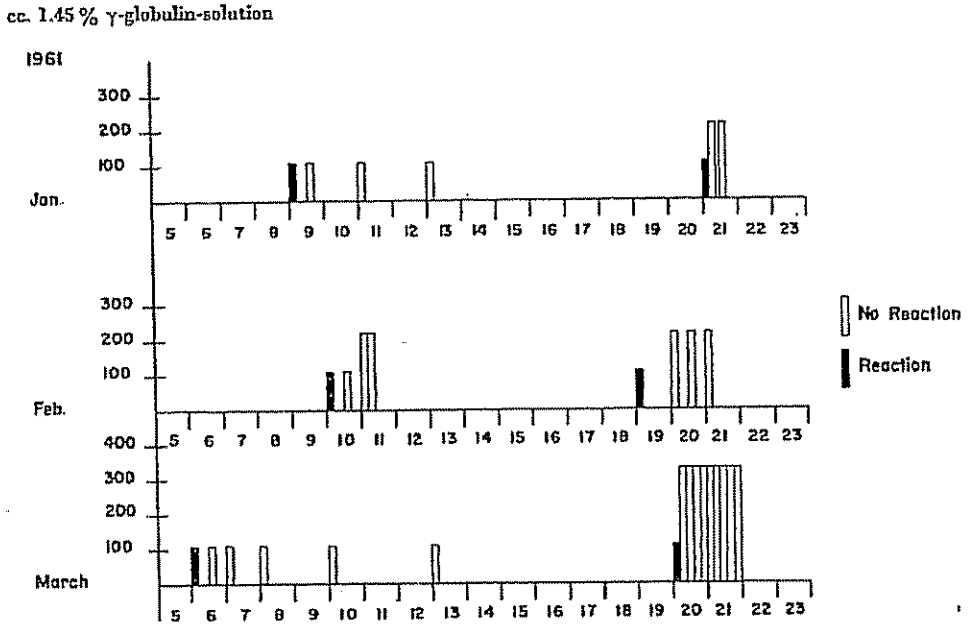
The *second phase* usually begins one or two hours after infusion with chills and a slight rise of temperature. The "sensitive" patients are pale and complain of fatigue. Often, especially in the standard test, subjects show only one or a few of the symptoms. Most frequent are: lumbar pain, oppression and flushing in the first phase, and slightly elevated body temperature in the second phase.

In severe reactions, which were rare in the standard test and, with one exception, occured only in patients with antibody deficiency syndrome, the course is much more violent, being reminiscent of anaphylactic shock. There is sudden onset of dyspnea, oppression, nausea and vomiting, lumbar pain and, rarely, circulatory collapse with loss of consciousness; this is immediately followed by a rise in temperature up to 39–40° C and chills. Even in severe reactions the symptoms usually disappear within 3 to 4 hours.

Further study of the possible cause of intolerance phenomena was carried out in 12 proved "sensitive" and an equal number of non-reacting control subjects.

If a "sensitive" patient is given several γ-globulin infusions at brief intervals – in the standard dosage – the following phenomenon may be observed: Table II. When the side effects from the first infusion have disappeared, the *patient* is *refractory to subsequent infusions*, at least for a certain period. Table II shows that after the initial reaction the "sensitive" patient can be given relatively large quantities of γ-globulin intravenously without any trouble. For example a "sensitive" patient (see Table II) received 45 g γ-globulin intravenously

TABLE II



between March 20 and 21 (i. e. approximately 300 ml of the standard 16 % γ-globulin) without showing the least side effects, apart from the initial reaction.

The refractory phase generally lasts 4 to 5 days. It occurs consistently once the initial infusion has brought about a full reaction, varying in degree for each patient. Partial reactions lead to only partial tolerance. The phenomenon will be discussed in a later section.

(2) Laboratory Studies

In extensive laboratory studies, the liberation of histamine or serotonin, as well as fibrinolytic processes, has been largely eliminated as possible causes of reactions. The histamine content of plasma before and after standard infusion was determined by the superfusion technique of Gaddum. In clinical trials the administration of histamine and serotonin antagonists did not lead to any significant reduction of intolerance phenomena [4].

The behavior of serum complement may afford a clue to the mechanism of the reaction: following a γ-globulin infusion a decrease in total complement was detected in all patients. As a rule, the decrease is much more pronounced in "sensitive" than in "non-sensitive" patients. The decrease was found to lie in all the components of the complement system. It should be mentioned that no significant drop in total complement has ever been detected after intramuscular administration of γ-globulin.

There are two possibilities to explain the decrease of serum complement after intravenous administration of γ-globulin: a complement-fixing antigen-antibody reaction or a non-specific consumption of complement by the γ-globulin preparation. It is well-known that γ-globulin preparations fix complement *in vitro*.

Table III shows that different batches of γ-globulin exhibit varying anticomplementary activity *in vitro*. It was greatest in sample Mg 24/25, which was slightly denatured while the ampoule was melted.

TABLE III

Anticomplementary Properties of Various γ-Globulin Preparations

γ-Globulin	Complement % inactivation*
Mg 24/25	74
Mg 24/25	50
Mg 20	44
Mg 18	41
Mg 22	49
Mg 35	43
MP 5905	31

* The figures indicate the amount of complement inactivated by different preparations of γ-globulin expressed in percent of the complement added in form of guinea-pig serum.

The greater anticomplementary activity in such cases is due to the occurrence of aggregated γ-globulin. The complement-binding mechanism of such aggregated particles is taken to be identical with that of antigen-antibody complexes [5].

(3) Removal of Anticomplementary Activity (AC Activity)

(a) By ultracentrifugation. In the belief that complement-fixing aggregates may also be formed in the process of plasma fractionation, attempts were made to detect and, if possible, to remove them by centrifugation. Standard γ-globulin (100 ml of a 16% solution) was centrifuged for 7 hours at 40,000 g in a model L Spinco ultracentrifuge. Four equal fractions were then separated with a syringe: a top, middle and bottom layer, plus a residue. The top and bottom layers, and the residue, were adjusted to 10% protein concentration, and then tested for AC activity.

Table IV shows that anticomplementary activity is greatest in the residual layer. The AC activity therefore appears to be associated with a heavy globulin fraction. The sedimentation diagram of the residue shows the following composition: a main gradient of over 93%, with a sedimentation constant of 6–7 S and approximately 5% of an 8–9 S component; the normal 19 S component is absent, but a heavy component of 30 to 40 S occurs in a concentration not exceeding 2% of

TABLE IV

Anticomplementary Properties of Various γ-Globulin Fractions Obtained by Ultracentrifugation

Fraction	Complement % inactivation*
"top"	44%
"bottom"	62%
"residue"	81%
control	0%

Sedimentation diagram of the "residue" fraction

Component of 6–7 S = approx. 93%
Component of 8–9 S = approx. 5%
Component of 30–40 S = approx. 2%

* Complement inactivation expressed as in Table III.

the total protein. There is reason to believe that this heavy component consists of aggregated γ-globulin formed in the process of plasma fractionation, and that it is responsible for the increased AC activity of the residual fraction. Similar runs of ultracentrifugation were carried out at 100,000 g. Under these conditions the top fraction consits exclusively of the 6–7 S component, without any contamination of the 8–9 S component.

(b) Chemical disaggregation. Reducing agents such as thiols are known to disaggregate certain macroglobulins into 6 S fragments by splitting disulfide bonds. Standard γ-globulin solutions were therefore treated with cysteamine at a concentration of 0.2 M and dialysed against buffered saline. No significant decrease of AC activity was observed. If the reduced globulin was treated with an equal volume of 0.2 M iodoacetamide, the AC activity disappeared. This experiment shows that the dissociation of disulfide bonds is reversible unless the newly formed sulfhydryl groups are blocked. Iodoacetamide alone at a concentration of 0.1 M only partially reduced the AC activity of γ-globulins (Table V).

Since iodoacetamide is toxic, experiments were carried out to block sulfhydryl groups with cystamine, the oxydation product of cysteamine. As shown by *Wilbrandt et al.* [7], cystamine may react with proteins in the presence of trace amounts of sulfhydryl groups to form mixed disulfides.

Proteolytic enzymes have been widely used for manufacturing commercial antibody preparations. The fragments obtained – though

TABLE V

Effect of Reducing Agents on Anticomplementary Activity of Human γ-Globulin *

HGG %	Treated for 6 hours at 20° C with cysteamine M	iodoacetamide M	cystamine M	AC activity*
7	–	–	–	100
7	0.1	0.1	–	<2
7	0.1	–	–	44
7	–	0.1	–	32
7	0.1	–	0.05	<5

* The figures indicate the amount of complement inactivated by chemically modified γ-globulin, expressed in percent of the AC activity of the non-treated original γ-globulin preparation. AC activity was measured according to *Kabat and Mayer* [6].

antigenically less active – still combine with antigens and diffuse more rapidly than the original molecules. Three fragments have been isolated and characterized from papain digested γ-globulin by *Porter* [8]. *Nisonoff* [9] and *Isliker* [7] isolated 5–6 S fragements from pepsin and trypsin digests which could be further split by reducing agents (mercaptoethylamine, sodium borohydride) into 3–4 S fragments.

In the present study, 32 mg pepsin (*Worthington, N. J.*) were dissolved in 5 ml isotonic saline and added to 20 ml of a 16% γ-globulin solution. The pH was adjusted to 4.0 with 0.1 N hydrochlorid acid and the resulting solution was incubated at 37° C. Samples were withdrawn after 1, 3, 6 and 20 hours and adjusted to pH 7, and a final protein concentration of 6%.

Using a pepsin/globulin ration of 1:100, nearly all AC activity had disappeared after 3 hours incubation and was non-existing after 20 hours.

The main components in the peptic digest displayed sedimentation constants of 5 and 3.5 S which were entirely deprived of AC activity. A main disadvantage consists in the fact that such fragments may be eliminated through the kidney, a finding which has been confirmed when a commercial preparation was infused which had a similar composition as ours ("Gammavenin"; Behringwerke, containing about 80% of a 5.1 S, 15% of a 2.4 S and 5% of a 7 S component *. Other samples digested with pepsin and further treated with reducing agents such as cysteamine were also deprived of AC activity [7]. Because of their even smaller molecular size and their loss through the kidney, these latter preparations have not been tested in patients.

We have therefore studied *procedures which would modify human γ-globulin without degradation*. The ratio pepsin to globulin was first changed from 1/100 to 1/1000, 1/10,000 and 1/100,000. It was found that the highest pepsin dilution would still decrease AC activity to negligeable values. Even more important was the finding that the control tube in which γ-globulin had been incubated at pH 4 for 17 hours without addition of pepsin was deprived of AC activity.

The effect of pH on the AC activity of γ-globulin has been recorded in Table VI. A nearly linear dependance can be observed below pH 7, but a pH as low as 4 and an incubation time of 6 hours at 37° C are required to deprive γ-globulin entirely of its complement-fixing properties. Below pH 3.8 a complete removal of AC properties was

* We are greatly indebted to Prof. Dr. *H. E. Schultze*, Behringwerke, Marburg/Lahn, for a generous gift of "Gammavenin".

TABLE VI

Effect of pH and Time of Incubation on AC Activity of Human γ-Globulin*

pH	1 h	3 h	6 h	20 h
6	–	90	62	41
5.5	–	71	50	–
5	71	–	(76)	21
4.5	36	–	25	5
4	25	<5	<2	<2
3.5	<2	<2	<2	<2
3	<2	<2	<2	–

* Samples of 6% γ-globulin (lot 47) were incubated at 37° C with increasing amounts of 0.1 N HCl and readjusted to pH 7 after 1, 3, 6 and 20 hours. AC activity is expressed as in Table V.

obtained within 3 hours. However, upon readjusting the pH from values below pH 3.8 to 7 distinct opalescence was observed and a slight precipitate was formed after standing for several weeks.

The effect of the time of incubation is recorded in the same table. The minimum duration required to remove AC activity largely depends on pH and temperature. As a rule, increasing the time of incubation has the same effect as lowering the pH. However, under certain conditions incubation for periods over 6 days at 37° may bring about an increase of AC activity due to the formation of aggregated γ-globulins.

The effect of temperature on the AC properties of γ-globulin has been studied at different pH values. Incubation at 20° C and 4° C at pH 4 is only partially effective. In order to reduce AC activity at 4° C a pH of 3 is required. Such a drastic treatment, however, will cause gelation of the protein unless it is exposed to acid for short periods only. Thus, incubation for 15 minutes at pH 3 and 4° C was sufficient to remove AC activity, whereas 6 minutes incubation did not suffice. However, even at these low temperatures a slight precipitation occurred upon readjustment of the pH to neutrality. The precipitate obtained under these conditions did not display AC activity.

Systematic variation of the 3 parameters: pH, time of incubation and temperature resulted in *optimal conditions between pH 3.8 and 4.0 and 24 hours incubation at 37° C.* However, certain lots of γ-globulin proved resistant to the latter conditions and pH values as low as

3.5 had to be resorted to in order to remove the last traces of AC activity. In the presence of 0.3 M glycine only a slight opalescence appears upon readjustment of the pH to neutrality. Because of these variations in the composition of "standard" γ-globulin it is *imperative* to test each new lot for AC activity before it is released for clinical studies.

(4) The Clinical Trials

A group of 20 "sensitive" patients was tested for tolerance to intravenous application of the various products derived from ultracentrifugation and from chemical disaggregation. All the patients examined consistently exhibited subjective and objective intolerance symptoms upon intravenous administration of standard γ-globulin. The different preparations derived from standard γ-globulin were adjusted to similar protein concentrations and infused under standard conditions. Table VII presents some typical examples.

Ultracentrifugation at 100,000 g yielded a top fraction which was well tolerated. Two patients showed no intolerance whatsoever. The top fraction obtained by centrifugation at 40,000 g was not tolerated significantly better than a standard γ-globulin solution. The centrifugal force of 40,000 g is insufficient to eliminate all the 8–9 S component which appears to play a major role for the AC activity and the incompatibility of the γ-globulins with sedimentation constants below 10 S.

Control experiments with the residue fraction, in which the heavy molecules of aggregated γ-globulin were accumulated, caused the recipients to produce the same symptoms as with standard γ-globulin.

Cysteamine-treated γ-globulin yielded no better results than the commercial product. This latter preparation, however, had not been treated with iodoacetamide, because of the toxicity of this sulfhydryl-blocking agent. No clinical studies are yet available with γ-globulin treated with cystamine.

γ-globulin digested by proteolytic enzymes was well tolerated. In this respect it is undoubtedly superior to all preparations. In order to test the "desensitizing" action of the various preparations, the patients received a test dose of standard γ-globulin (110 ml 1.45% standard γ-globulin, infused in 90 to 120 min.) 6–10 hours after the first infusion. The results are shown in Table VII in the "TT" (tolerance test) column. The well-tolerated, enzymatically treated γ-globulin preparation ("Gammavenin") displayed no desensitizing action. On

TABLE VII

TABLE VII

Clinical Compatibility of Different γ-Globulin Preparations in "Sensitive" Individuals

γ-Globulin preparations administration by i.v. route	L. J. Fr. & 1952 ADS (a–γ) Symptoms			T, Fr. Q 1907 ADS (hypo-γ) Symptoms			H. O. & 1919 ADS (hypo-γ) Symptoms			S. H. U. & 1941 ADS (hypo-γ) Symptoms			F. E. & 1933 Osteomyelitis Symptoms		
	subj.	obj.	T. T.	subj.	obj.	T. T.	subj.	obj.	T. T.	subj.	obj.	T.T.	subj.	obj.	T.T.
Standard (110 ml 1.45% infused in 90')	++	++	•	++	++	•	++	+	•	+	+	•	+	+	•
UC. 100,000 g "top"-fraction (110 ml approx. 1.5% infused in 90')	•	(+)	(+)	–	–	–	•	•	(+)	•	•	(+)	–	–	–
UC. 40,000 g "top"-fraction (110 ml approx. 1.5% infused in 90')	+	++	•	–	–	–	+	+	•	(+)	•	•	•	(+)	•
Disaggregated with Cysteamine (110 ml approx. 1.5% infused in 90')	+	++	•	++	++	•	+	+	•	(+)	(+)	•	(+)	(+)	•
Degraded with Proteolytic Enzyme ("Gammavenin") (110 ml 2.5% infused in 90')	•	•	++	(+)	•	++	•	•	++	•	•	+	•	•	(+)

++ = pronounced symptoms; + = slight symptoms; (+) = trace of symptoms; • = no symptom; TT = Tolerance test.

TABLE VIII

Clinical Compatibility of pH 4 Exposed γ-Globulin in "Sensitive" Individuals

γ-Globulin preparations administration by i.v. route	D.B. ♂ 1940 ADS (hypo-γ) subj.	D.B. ♂ 1940 ADS (hypo-γ) obj.	D.B. ♂ 1940 ADS (hypo-γ) T. T.	E. P. ♂ 1947 ADS (normo-γ) subj.	E. P. ♂ 1947 ADS (normo-γ) obj.	E. P. ♂ 1947 ADS (normo-γ) T. T.	H. O. ♂ 1919 ADS (hypo-γ) subj.	H. O. ♂ 1919 ADS (hypo-γ) obj.	H. O. ♂ 1919 ADS (hypo-γ) T. T.	L. J. F. ♂ 1922 ADS (a-γ) subj.	L. J. F. ♂ 1922 ADS (a-γ) obj.	L. J. F. ♂ 1922 ADS (a-γ) T. T.	S. H. U. ♂ 1941 ADS (hypo-γ) subj.	S. H. U. ♂ 1941 ADS (hypo-γ) obj.	S. H. U. ♂ 1941 ADS (hypo-γ) T. T.	S. O. ♂ 1898 ADS (hypo-γ) subj.	S. O. ♂ 1898 ADS (hypo-γ) obj.	S. O. ♂ 1898 ADS (hypo-γ) T. T.
Standard (110 cc 1.45%) infused in 90')	++	++	*	+	+	*	++	+	*	++	++	*	+	+	*	++	++	*
γ-Globulin "pH 4" (110 cc approx. 1.5% infused in 90')	*	*	(+)	*	*	(+)	(+)	*	*	*	*	*	*	*	*	*	*	*

++ = pronounced symptoms; + = slight symptoms; (+) = trace of symptoms; * = no symptoms; TT = tolerance test.

the other hand, after receiving the incompatible, standard γ-globulin, "sensitive" patients were found to be refractory.

In our trials the main enzymatically treated preparation used was "Gammavenin" from the Behring Werke, which is now undergoing clinical trial but is not yet on the market. More than 20 "sensitive" patients were given over 50 "Gammavenin" infusions; reactions, in the form of subjective symptoms or a slight rise in temperature, were exceedingly rare. As a rule, no "sensitive" patients displayed any side-effects at all. However, as pointed out previously, this preparation is rapidly eliminated throug the kidney. The urine of a patient who had received 300 ml of "Gammavenin" (10% protein solution) was concentrated and tested for γ-globulin fragments by immunological techniques. Large amounts of γ- and β-globulins were found in this urine. No proteinuria had been observed prior to the infusion of the preparation.

γ-globulin obtained by incubation at pH 4 was tolerated like "Gammavenin". It must be reemphasized that each new lot of pH treated γ-globulin must be carefully tested for AC activity. When the latter persists, the lot must be reincubated at pH 4 or less and 37° C until it is no longer anticomplementary. No γ-globulin fragments were observed in the urine and the pH 4 treated γ-globulin had not lost its ability to desensitize "sensitive" patients. The results obtained in the first 6 "sensitive" patients are recorded in Table VIII. The pH 4 exposed γ-globulin was well tolerated by all 12 tested, otherwise "sensitive" patients. Only one subject had a slight rise in temperature (0.5° C) 1–2 hours after the infusion. In contrast to the behavior after application of "Gammavenin", the patients were largely refractory in the tolerance test. Table IX shows the behavior of serum complement before and after the intravenous application of the different γ-globulin preparations tested in this study. A significant decrease of the serum complement activity was observed only after administration of the nonmodified standard preparation.

(5) Discussion

Some indirect conclusions about the cause of intolerance reactions in "sensitive" patients can be drawn from the different behavior of patients with an antibody deficiency syndrome (ADS) to standard γ-globulin and physically or chemically modified γ-globulin.

(1) Patients with an ADS are unable to form antibodies. It is therefore unlikely that the reactions observed in these patients after

TABLE IX

Complement Consumption in Patients treated with Different Preparations of Human-γ-Globulin

Patient Control Nr.	Reaction in Standard-test	C′-Titer before i.v. Application of γ-globulin	C′-Titer after i.v. Application of γ-globulin	γ-Globulin* preparations
1	+	155	100	Standard-γ-globulin
2	+	75	64	
3	+	170	106	
4	+	128	85	
5	+	85	41	
6	+	85	60	
7	+	79	48	
8	−	105	100	
9	−	79	74	
10	−	69	54	
11	−	80	75	
12	−	93	84	
1	+	140	144	enzymatically degraded γ-globulin
2	+	64	56	
3	+	125	112	
4	+	200	200	
1	+	46	37	pH 4-exposed γ-globulin
2	+	42	42	
3	+	41	64	
13	+	22	25	
14	+	37	34	

Legend: Patients with the control Nr. 1–7 and 13–14 displayed an ADS; patients with Nr. 8–12 showed no immunological disturbances.

* The determination of complement has been performed separately for each γ-globulin preparation.

intravenous γ-globulin injection are due to a direct antigen-antibody reaction, the recipient's antibodies reacting with antigens present in the γ-globulin administered.

(2) The assumption of an inverse antigen-antibody reaction i.e. antigens in the recipient organism reacting with antibodies of an administered γ-globulin preparation would explain the striking observation that regular and severe reactions are almost entirely confined to ADS patients. In these patients the humoral antibody

defense barrier is impaired; germs and their breakdown-products may more easily be resorbed from the respiratory and gastrointestinal tract than in normal individuals.

(3) γ-globulin, modified either by enzymatic or acid treatment, has a similar antibody spectrum as standard γ-globulin and causes no reaction in contrast to the nonmodified standard preparation [10]. We must therefore assume that *the intolerance is not a direct effect of an antigen-antibody reaction. It is in some way linked to the AC activity of γ-globulin* which varies to a large extent from one lot to another. Certain preparations have been shown to contain high-molecular aggregated γ-globulins which fix complement by a similar mechanism as antigen-antibody complexes [5, 11, 12].

However, γ-globulin deprived of aggregated 30–40 S γ-globulin by ultracentrifugation still exhibits AC activity and may not be always tolerated by sensitive patients. Our results indicate that 8–9 S γ-globulins are also anticomplementary and incompatible for intravenous administration. Enzymatic treatment, exposure to reducing agents or to pH 4 at 37° C affect the complement binding sites either by a disruption of covalent bonds or a distortion of the γ-globulin molecule.

Preliminary experiments indicate that the fragments obtained by enzymatic digestion will form complexes with antigen which are unable to fix complement. On the other hand, antibody exposed to pH 4 – although deprived of AC activity – will fix complement in presence of the homologous antigen (Table X). These difference are of considerable importance since bacteria and other cells can only be lysed when their complexes with antibody are able to fix complement.

A most striking effect is the fact that a non-compatible γ-globulin infusion causes "sentitive" patients to become temporarily tolerant to subsequent intravenous doses of γ-globulin. In contrast, enzymatically digested γ-globulin (such as "Gammavenin") does not cause subjects to become "desensitized". A subsequent standard infusion brings about typical, fullstrength intolerance phenomena. In this respect γ-globulin exposed to pH 4 behaves like standard γ-globulin and the question as to the reason for this "desensitizing" effect was raised. Experiments conducted in collaboration with *Cruchaud and Frei* [13] on the *in vitro* fixation of I^{131}-labelled γ-globulins to fresh human liver tissue have shed some light on this problem. It was shown that the untreated standard preparation was fixed to a much larger extent than the digested γ-globulin. The preparation exposed to

TABLE X

Fixation Behavior of Different Preparations of Human-γ-Globulin

γ-Globulin preparations	Fixation of C′ *per se*	Fixation of C′ in the presence of antigen**	Fixation on tissues***	Loss through the kidney
Native	++	+++	+++	−
Enzymatically degraded	*	*	+	+
Exposed to pH 4	*	++	+++	−

* No fixation of complement.

** The fixation of complement was measured in the system sheep erythrocytes-rabbit hemolytic antibody.

*** These experiments were kindly performed by Drs. *S. Cruchaud and P. Frei* by measuring the fixation of I^{131}-labelled γ-globulin on human liver tissue [13].

pH 4 was fixed similarly or slightly less than untreated γ-globulin. Thus, it would appear that "desensitization" may only be accomplished by preparations which are fixed to tissues. Desensitization may last as long as the tissue "receptors" are saturated with γ-globulins.

In this respect, the fact that patients suffering from an ADS are most susceptible to reactions after intravenous administration of γ-globulin, deserves further consideration. This particular behavior of ADS patients could be explained in terms of a tissue-bound reaction with γ-globulins. The tissue "receptors" of AD patients are likely to exhibit a higher affinity for γ-globulin and may therefore produce more serious reactions.

It appears from Table X that untreated γ-globulin has a high affinity for tissues as well as for complement, whereas the pH 4 exposed γ-globulin may still fix to tissues without displaying AC activity. Further experiments are in progress to test if there is a causal relationship between these *in vitro* phenomena and the different *in vivo* behavior of the enzymatically degraded and the pH 4 exposed preparations.

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Summary

Intravenous administration of standard human γ-globulin can lead to untoward reactions.

This is due to at least one *endogenous* and an *exogenous* factor.

The "*endogenous*" factor is revealed by the finding that certain subjects only – especially patients with an antibody deficiency syndrome – react to intravenous infusions of γ-globulin administered under standard conditions.

The "*exogenous*" factor resides in the γ-globulin preparation itself and is related to its anticomplementary activity.

The "*endogenous*" factor can be temporarily eliminated by "desensitizing" the patient; the "*exogenous*" factor may be eliminated by ultracentrifugation, enzymatic breakdown of the γ-globulin, or other treatment involving exposure to low pH values.

Upon clinical trial, the preparations obtained by enzymatic degradation or by exposure to pH 4 at 37° C displayed the best tolerance after intravenous application. Enzymatically degraded γ-globulin is partially eliminated by the kidney and has no desensitizing effect. On the other hand, pH 4 exposed γ-globulin does not pass through the kidney and is still capable of desensitizing "sensitive" individuals.

Résumé

L'administration intraveineuse de γ-globuline humaine peut provoquer chez l'homme des réactions sérieuses.

Ces phénomènes d'intolérance sont dus à l'existence au moins d'un facteur *endogène* et d'un facteur *exogène*.

Le facteur "*endogène*" se manifeste en ce sens que seul certains malades – spécialement ceux souffrant d'un syndrome par carence d'anticorps – tolèrent mal l'injection intraveineuse de γ-globuline.

Le facteur "*exogène*" est associé à la γ-globuline même et est en relation directe avec son action anticomplémentaire.

Le facteur "*endogène*" peut être éliminé temporairement par une désensibilisation du malade; le facteur "*exogène*" peut être éliminé par ultracentrifugation, par dégradation enzymatique de la γ-globuline ou par d'autres traitements tels qu'une exposition prolongée à des acides très dilués.

Ce sont les préparation obtenues par dégradation enzymatique ou par l'action d'acides dilués qui sont le mieux tolérées lors de l'injection intraveineuse.

La γ-globuline digérée sous l'action d'un enzyme est éliminée partiellement par les reins et n'exerce aucune action désensibilisante. Par contre, la γ-globuline exposée à un pH de 4 ne passe pas le seuil rénal, et sa capacité de désensibiliser des individus sensibles, persiste

Zusammenfassung

Die intravenöse Applikation von menschlichem Standard-γ-Globulin kann zu schweren Zwischenfällen Anlaß geben.

Diese Unverträglichkeit beruht zumindest auf einem *exogenen* und einem *endogenen* (individuellen) Faktor.

Der "*endogene*" (individuelle) Faktor äußert sich darin, daß nur gewisse Individuen – in der Regel handelt es sich dabei um Patienten mit einem Antikörpermangelsyndrom – auf eine i.v. γ-Globulingabe mit Unverträglichkeitserscheinungen reagieren.

Der "*exogene*" Faktor liegt im γ-Globulinpräparat; er steht mit dessen antikomplementärer Wirkung in direkter Beziehung.

Es ist möglich, den "*endogenen*" Faktor vorübergehend durch "Desensibilisierung" des Patienten (mit Standard-γ-Globulin) auszuschalten. Der "*exogene*" Faktor kann entweder durch Ultrazentrifugierung, durch enzymatische Verdauung, oder durch bloße Ansäuerung (pH 4) eliminiert werden.

Bei der klinischen Prüfung erwiesen sich diejenigen Präparate als gut verträglich, die durch enzymatische Verdauung oder durch Ansäuern gewonnen wurden. Enzymatisch abgebaute γ-Globuline gehen teilweise durch die Niere verloren; sie besitzen außerdem keine desensibilisierende Wirkung. Demgegenüber erscheint das durch Ansäuern gewonnene Präparat nicht im Urin des Patienten. Es verhält sich auch in bezug auf seinen desensibilisierenden Effekt wie Standard-γ-Globulin.

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EXHIBIT 13

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Failure of a human immunodeficiency virus (HIV) immune globulin to protect chimpanzees against experimental challenge with HIV

(passive immunization/neutralizing antibody/human immunodeficiency virus vaccine)

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ABSTRACT To assess the possible efficacy of passive immunization against human immunodeficiency virus (HIV) an immune globulin was prepared from plasma of HIV-seropositive donors selected to be among those having the top 12.5% of virus-neutralizing antibody titers. The immune globulin was treated with pepsin to render it intravenously tolerable. The preparation, which we termed HIVIG, neutralized 100 tissue culture 50% infective doses ($TCID_{50}$) of HIV at an average dilution of 1:1000 in neutralization tests *in vitro*. During preparation HIVIG was subjected to virus inactivation and removal procedures that in theory resulted in a reduction in HIV infectivity by a factor of 10^{25}. At a dose of 9–10 ml/kg of body weight both the virus-inactivated source plasma and the final immunoglobulin preparation were noninfective and without adverse effect in two chimpanzees. Two chimpanzees inoculated intravenously with HIVIG at 1 ml/kg and two inoculated with 10 ml/kg were challenged intravenously 1 day later with 400 $TCID_{50}$ of the same strain of HIV ($HTLV\text{-}III_b$) used in neutralization assays *in vitro*. All animals became infected. Incubation periods to virus isolation (by cocultivation with human mononuclear cells) in HIVIG recipients did not differ significantly from the incubation period seen in a control animal that received a normal anti-HIV-free immunoglobulin. These findings may have implications for understanding the failure of experimental vaccines to protect against HIV challenge in chimpanzee experiments.

Active immunization may ultimately provide the most effective means for halting the world-wide epidemic of infection with human immunodeficiency viruses (HIVs). However, so far, envelope protein-based experimental vaccines have failed to protect chimpanzees against challenge with live virus (1–3). In these trials good cell-mediated immunity as assessed by *in vitro* blastogenesis and T-cell cytotoxicity was induced; however, only relatively low or no neutralizing antibodies were present at the time of challenge. In the present study we evaluate the possibly protective effect of high neutralizing antibody levels produced by passive administration of human neutralizing antibodies.

Studies on seropositive male homosexuals revealed that 92.7% had neutralizing antibodies, with a median titer of about 1:50 and with 12.5% having titers of 1:128 or greater (4). By selection of donors from the high-titer group it appeared feasible to prepare a hyperimmune globulin that would allow the protective effect of high-titer neutralizing antibody to be investigated. Such a preparation might have clinical utility in prevention of infection in certain settings such as after accidental or maternal–infant exposures.

Precedent exists for the use of passive antibody, even when given days after exposure, to prevent retroviral disease. In the case of Friend leukemia virus, administration of passive anti-envelope antibodies 1 week after virus inoculation completely protected mice, while controls receiving normal IgG all died 8–12 weeks after inoculation (5). Similar results were obtained in the feline leukemia virus model (6).

MATERIALS AND METHODS

Preparation of HIV Immune Globulin. Source plasma was obtained by plasmapheresis from HIV-seropositive donors free of signs of acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) who had neutralizing antibody titers of ≥1:128.

To eliminate the possibility that an immunoglobulin made from HIV-infected plasma could transmit HIV infection, we made use of our experience in developing virus-sterilized coagulation factors by application of solvents and detergents (7–9). Pooled plasma was first exposed to tri(*n*-butyl) phosphate at 2% for 4 hr at 37°C. This step has been found to inactivate ≥4.2 $\log_{10}$[tissue culture 50% infective dose ($TCID_{50}$)] units of HIV, ≥6 $\log_{10}$[chimpanzee 50% infective dose (CID_{50})] units of hepatitis B virus, and ≥5 $\log_{10}CID_{50}$ units of non-A, non-B hepatitis virus (data not shown). This preliminary virus-inactivation step was introduced primarily to provide safety to the workers during the Cohn fractionation procedure. The tri(*n*-butyl) phosphate-treated plasma was fractionated by the Cohn–Oncley cold ethanol procedure (10). This procedure has been shown to inactivate or remove at least 12 $\log_{10}$ units of HIV infectivity (11). Purified gamma globulin (fraction II) was then treated with 0.3% tri(*n*-butyl) phosphate and 1% Tween 80 at 25°C for 6 hr, a step that has been found to inactivate ≥3.8 $\log_{10}TCID_{50}$ units of HIV and ≥5 $\log_{10}CID_{50}$ units of non-A, non-B hepatitis virus (data not shown). Finally, to prepare intravenously tolerable immunoglobulin the globulin was held at pH 4.1 for 21 hr at 37°C in the presence of 350 units of pepsin per gram of protein. This step has been found to inactivate ≥4.35 $\log_{10}TCID_{50}$ units of HIV within 2 hr (data not shown). Thus, altogether an

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Abbreviations: HIV, human immunodeficiency virus; HIVIG, HIV immune globulin; $TCID_{50}$ tissue culture 50% infective dose; CID_{50}, chimpanzee 50% infective dose; ADCC, antibody-dependent cell-mediated cytotoxicity.

†To whom reprint requests should be addressed.

aggregate HIV inactivation and removal process efficacy of as much as ≥25.3 $\log_{10}TCID_{50}$ units may result from the virus inactivation and processing steps employed to prepare HIV immune globulin (HIVIG).

As shown by $NaDodSO_4$/PAGE analysis and gel exclusion chromatography this procedure produces largely undegraded 7S gamma globulin.

Two lots of HIVIG have been prepared to date. The first lot (HV101) was derived from 31 liters of plasma representing 24 donors; the second lot (HV102) was derived from 32 liters of plasma obtained from 17 donors. Plasma for these lots was obtained from donors having required levels of neutralizing antibody at the New York Blood Center, at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, and at the National Center for Blood Transfusion in Paris.

The final preparation of HIVIG (lot HV102) was made up to a protein concentration of about 5%, 97–98% of which was IgG. Gel exclusion chromatography revealed 86% 7S monomer, 0.3% dimer, and 14% fragments. The anti-HIV neutralizing antibody titer was found to vary between 1:500 and 1:3200 (mean 1:1000) in tests carried out in the New York Blood Center laboratories, the Central Laboratories of the Netherlands Red Cross Blood Transfusion Service, and the Institut Pasteur with an MT-4 cell-based assay (12). The anti-HIV ELISA titer (last dilution positive by DuPont assay) was 1:640,000; assessment of the dilution giving an A_{492} of 0.5 gave a titer of 1:72,443.

Chimpanzee Studies. Adolescent or adult chimpanzees (*Pan troglodytes*) previously exposed to non-A, non-B hepatitis virus were used in these studies. They were housed under conditions exceeding the minimum requirements outlined in the National Institutes of Health Guide for the Care of Laboratory Animals. Animals were bled at 2-week intervals after tranquilization with ketamine hydrochloride at 5 mg/kg.

HIV-Neutralizing Antibody Tests. Serial dilutions of heat-inactivated sera were made in microtiter plates in RPMI 1640 medium/20% fetal calf serum, containing Polybrene at 2 μg/ml and 100 $TCID_{50}$ of the HTLV-III_b strain of HIV per 100 μl. After incubation for 2 hr at 4°C, H9 cells were added to bring the final concentration to 8×10^5 cells per ml in a total volume of 150 μl per well. Cultures were fed by addition of 25 μl of medium on days 4, 7, and 10. On day 14 75 μl of supernate was removed for reverse transcriptase assay as described (4). The titer was calculated as geometric mean of two or three duplicate assays and is expressed as the highest dilution in which reverse transcriptase assay is less than 3 times the mean of virus-free negative control cultures (4).

Serologic Assays. Anti-HIV antibodies were measured by ELISA with kits provided by DuPont. Serial dilutions were made in diluent provided by the manufacturer. The dilution giving an A_{492} of 0.5 was interpolated from the linear portion of the curve relating absorbance and dilution. Immunoblots ("western blots") were prepared with strips provided by Bio-Rad in accordance with the manufacturer's instructions.

Virus Assays and Isolation. At 2-week intervals 10^7 chimpanzee peripheral mononuclear cells (PBMC) were cultured with 10^7 normal human PBMC as described by Gallo *et al* (13). Weekly for 4 weeks the supernatants from the cocultures were assayed for p24 antigen (Abbott Laboratories kit) and attempts were made to isolate virus from the cocultures (13).

RESULTS

Evaluation of Safety of HIVIG. To confirm the safety of HIVIG experimentally, two chimpanzees were inoculated intravenously with the first lot of HIVIG at 10 ml/kg of body weight and two other chimpanzees were inoculated with the tri(*n*-butyl) phosphate-treated source plasma at 9.1 ml/kg. The latter group provided a more stringent test of safety as the material inoculated had been exposed to only the first of the four virus inactivation and removal steps undergone by the final preparation. These animals were studied by taking blood samples 1, 3, 7, and 14 days after inoculation and then biweekly for 9 months. Serum was saved for HIV serology; leukocyte and platelet counts and differential counts were carried out; heparinized blood was saved for fluorescence-activated cell sorting analysis; and peripheral blood lymphocytes were isolated by Ficoll/Hypaque centrifugation and cryopreserved in liquid N_2 for virus isolation studies should these be indicated.

Anti-HIV detectable by ELISA against whole disrupted virions (DuPont) declined steadily with a half-life of 18 days characteristic of immunoglobulins injected into humans (data not shown). This reflected the near identity of chimpanzee and human immunoglobulins, as well as the intactness of the immunoglobulins. Further followup of anti-HIV levels revealed that they continued to decline steadily at the same rate until becoming undetectable at 4–5 months. This indicated that active infection did not occur during the 9-month follow-up period. No alterations in leukocyte counts, differential counts, T-cell subsets, or B-cell counts were observed (data not shown). This indicated that the autoantibodies reactive with B cells and T cells found in the serum of HIV-infected persons (14–16), and present also in HIVIG (data not shown), do not appear to have pathogenic significance in chimpanzees.

Evaluation of Protective Efficacy. Two experiments have been done in chimpanzees to evaluate the potential efficacy of HIVIG in preexposure prophylaxis of HIV infection by cell-free virus.

In the first experiment a 1 ml/kg dose of lot HV102 was administered to two chimpanzees (20 and 24 kg) intravenously. As a control, a third animal received 1 ml/kg intravenous injection of immunoglobulin devoid of antibodies to HIV, kindly donated by Biotransfusion (Lille, France). In the second experiment (see below) a 10-fold higher dose of HIVIG was used.

On the basis of plasma volume considerations, the expected dilution of HIVIG resulting from this injection was about 1:100 for the low-dose group and 1:10 for the high-dose group. On the basis of serologic assays carried out on HIVIG and on serum taken 24 hr after injection, the actual dilution in the low-dose group was somewhat greater but was about 1:10 in the high-dose group (Table 1). Animals receiving 1 ml/kg revealed an average titer (determined by the method given below Table 1) of anti-HIV by ELISA assay of 1:553, representing a 1:131 dilution from the HIVIG titer of 1:72,443.

On the day after receipt of globulins all animals were challenged by intravenous inoculation of a stock of HTLV-III_b HIV, which had been titrated in chimpanzees by inoculation of 10-fold dilutions into one or two animals per dilution. This stock was kindly provided by Larry O. Arthur and Peter Fischinger (National Cancer Institute). The stock is considered to contain 10^4 $TCID_{50}$/ml when titrated in H9 cells and an estimated 2.5×10^3 CID_{50}/ml. The challenge dose consisted of 1 ml of a 1:25.6 dilution in saline, which corresponded to 400 $TCID_{50}$ and an estimated 100 CID_{50}. Titration of this diluted challenge material *in vitro* on the day of challenge revealed a titer of 100 $TCID_{50}$/ml. Four subsequent assays at the Southwest Foundation revealed titers varying between 1:100 and 1:1200, with a mean for all assays of 1:540.

Inoculated animals were studied by biweekly anti-HIV ELISA assays, immunoblots, p24 antigen assays, and virus isolation attempts by cultivation with human peripheral blood lymphocytes.

Table 1. Anti-HIV titers, *In vivo*, 24 hr after administration of HIVIG

Sample	HIVIG dose, ml/kg	Anti-HIV ELISA titer*	Mean dilution from HIVIG	Anti-HIV ADCC titer†	Neutralizing antibody titer‡
HIVIG (HV102)	—	1:72,400	—	>1:200,000	1:944
Chimp X212	1	1:600	1:131	1:1,800	<1:20
Chimp X233	1	1:500		1:1,900	<1:20
Chimp X130	10	1:6,300	1:9 6	>1:20,000	1:354
Chimp X310	10	1:9,300		>1:20,000	1:178
Chimp 333§	10	1:7,400	1:10 9	ND	1:126
Chimp 334§	10	1:5,900		ND	1:89

*ELISA determinations for anti-HIV were done on sera taken 24 hr after administration of HIVIG with kits provided by DuPont in which the solid phase consisted of whole disrupted purified virions attached to the surface of microtiter plates. Samples were serially diluted in phosphate-buffered saline/0.02% Tween 20. The dilution corresponding to an A_{492} of 0.5 was then determined from the linear curve relating dilution and absorbance. Titers obtained by this method are 1/4th to 1/8th of those estimated on the basis of the last positive dilution; however, they are more accurate.

†Antibody-dependent cell-mediated cytotoxicity (ADCC) assays were performed with uninfected CD4+ cells adsorbed with gp120 as previously described (17). Titers are expressed as the serum dilution corresponding to a 50% reduction in maximal lysis. ND, not determined.

‡Geometric mean of four assays.

§Chimpanzees used in HIVIG safety test.

The results of the first efficacy trial are shown in Figs. 1 and 2. The control animal, chimpanzee X304, developed anti-p24 detectable on immunoblots on week 5, and by week 7 virus was isolated from cocultures. The treated animals, when tested at 24 hr, showed passive antibody reactive with all major HIV-associated bands; the antibodies persisted for various periods of time, depending on their respective concentrations. However, cocultivation revealed as soon as week 3 that chimpanzee X212 had become infected. This was confirmed by the reappearance of anti-p17 on week 7. Similarly, chimpanzee X233 revealed infection first by cocultivation on week 13 and anti-p17 detectable on immunoblots reappeared later (data not shown). This animal has since yielded HIV in all subsequent cocultivations.

Study of serial sera from these animals for anti-HIV ELISA titers (Fig. 2*A*) revealed appearance of antibody in the control animal at 5 weeks. At 9 weeks there was a fall in titer, perhaps reflecting the intensity of viral replication, followed by return to relatively high levels. The treated animals showed a gradual slowing of the rate of decline in antibody titer followed by a gradual rise in titer, again indicating that infection had occurred.

Clearly the 1 ml/kg dose of HIVIG failed to protect against a challenge of 400 $TCID_{50}$ of HIV. Therefore we undertook a second efficacy study, utilizing a 10-fold higher dose of HIVIG, given as a replacement for a corresponding volume of removed plasma, prior to challenge, and in addition second, third, and fourth doses of 1 ml/kg were scheduled at monthly intervals after challenge. To conserve chimpanzees no control was included in the second study, since the infectivity of the challenge stock, maintained at −70°C in the multiple aliquots supplied to us, had been confirmed.

The results of the second efficacy trial are summarized in Figs. 1*B* and 2*B*. As expected, immunoblots revealed large amounts of passive antibody reactive with all major HIV-associated bands. These precluded observation of the onset of seroconversion. However, cocultivation revealed that virus replication occurred in both treated animals, with virus isolation occurring for the first time on week 3 from chimpanzee X130 and on week 9 from chimpanzee X310. Because of the large amount of passive antibody, virus replication is indicated from serial anti-HIV testing (Fig. 2*B*) only by the fact that antibodies did not decline at the rate (half-life of 18 days) expected if no active antibody synthesis was occurring.

DISCUSSION

Fig. 1 shows the times of onset of viremia in the five animals taking part in the efficacy trials. The mean of the incubation periods of the animals treated with HIVIG at 1 ml/kg (8 weeks) and 10 ml/kg (6 weeks) were indistinguishable from the incubation period (7 weeks) in the control animal. Thus neither protection nor enhancement can be concluded. This is further supported by the 4-week incubation period observed in a chimpanzee inoculated with 400 $TCID_{50}$ and the 2- and 4-week incubation periods observed in two other chimpanzees inoculated with 40 $TCID_{50}$ of the virus stock used in the present study (L. O. Arthur, personal communication).

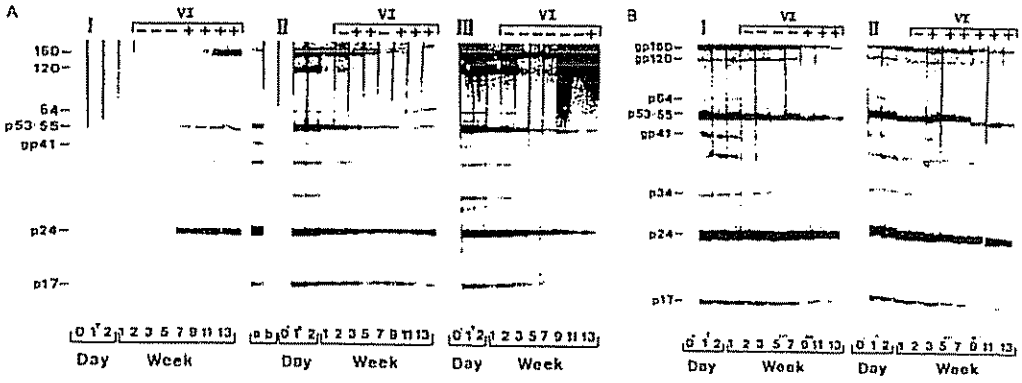

FIG. 1. Immunoblots and results of virus isolations (VI) from HIVIG- and control immunoglobulin-treated chimpanzees. (*A*) I, the control animal, X304; II and III, HIVIG (1 ml/kg)-treated chimpanzees X212 and X233, respectively. 0, antibody pattern on day 0 for control animal before immunoglobulin (1 ml/kg) infusion; 0^*, antibody pattern on day 0 for HIVIG-treated animals; $1^\dagger$, antibody pattern 1 day after infusion of HIVIG or immunoglobulin. (*B*) I and II, HIVIG (10 ml/kg)-treated chimpanzees X310 and X130, respectively. Symbols are identical to those in *A* except that each animal received booster injections of HIVIG (1 ml/kg) at weeks 5 and 9 (5^{**}, 9^{**}). A + for virus isolation indicates that virus was detected by p24 antigen assay.

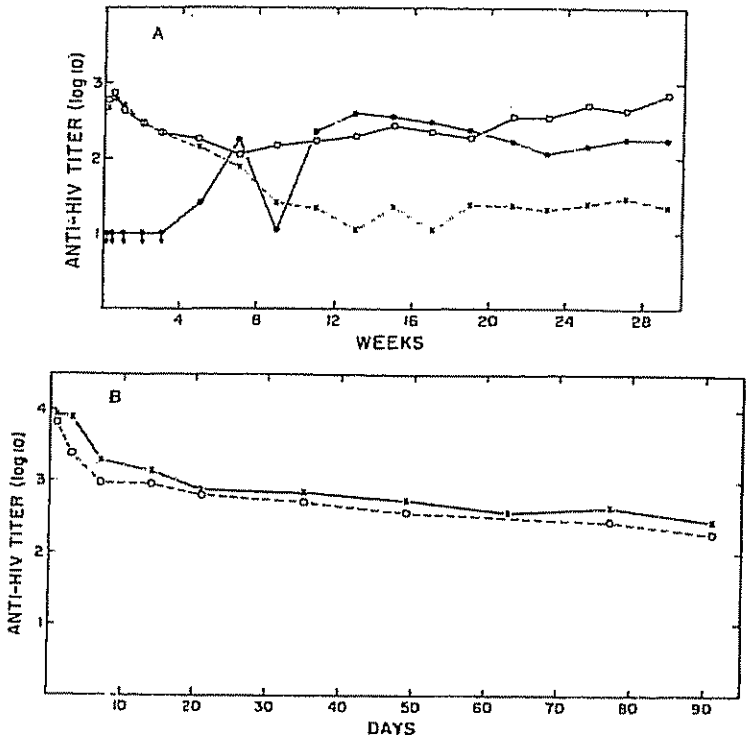

FIG. 2. Anti-HIV ELISA titers, measured by the method described in the legend to Table 1 on serial sera drawn from the chimpanzees. (*A*) First efficacy trial. ●, Control chimpanzee X304. ○, Chimpanzee X212 and ×, chimpanzee X233, each treated with HIVIG at 1 ml/kg. (*B*) Data from the second efficacy trial. ×, Chimpanzee X310 and ○, chimpanzee X130, each treated with 10 ml/kg.

The finding that even a level of neutralizing antibody approximately 100 times that required to neutralize 100 $TCID_{50}$ *in vitro* failed to neutralize 400 $TCID_{50}$ *in vivo* is surprising and has many important implications for attempts to prevent HIV transmission with experimental vaccines designed to elicit neutralizing antibody.

Several testable hypotheses can be considered in the search for an explanation of these findings.

First, it is possible that the *in vitro* neutralization assays used overestimate neutralizing capacity. Our assay (4) uses an incubation time of 14 days, which is sufficient for detection of 1 $TCID_{50}$ in virus titrations (18). It is possible that antibody-coated virus infects more slowly, and furthermore that spread of infection is slowed but not completely prevented by the presence of antibody. Thus "complete neutralization" in 14 days may actually reflect only partial neutralization. However, this appears unlikely, as continued incubation of neutralization assay cultures for 2 additional weeks did not result in appearance of virus detectable by reverse transcriptase in wells showing neutralization at 2 weeks (data not shown). Furthermore, washing of cells 4 hr after addition of virus–antibody mixtures did not greatly affect neutralizing antibody titers, indicating that the test measured actual neutralization rather than an interference with spread of virus due to residual antibody (data not shown).

Second, it could be that the treatments used to prepare an intravenously tolerable, virus-free immunoglobulin may have impaired its biological activity. This is unlikely, since the resultant globulin was predominantly intact 7S IgG, had a normal half-life *in vivo*, and showed intact neutralizing and ADCC activity. The latter in particular demonstrates intact Fc receptor binding activity.

Third, it is conceivable that HIV has an exceptionally high "resistant fraction" in kinetic neutralization curves. All viruses show a proportion of such neutralization-resistant virions, usually at a frequency of 10^{-3} or 10^{-4} (19). Perhaps due to its high rate of spontaneous variation (20) HIV may show a higher proportion of virions that are partially or completely resistant to neutralization by antibody. If this is the case it is likely that the viruses isolated from animals that were not protected by large amounts of neutralizing antibody in the above studies will show enhanced resistance to neutralization in comparison to the initially infecting challenge inoculum.

Fourth, it is possible that the high affinity constant ($\approx 10^9$ M^{-1}) for the association between HIV and CD4-linked receptors (21) results in successful competition with binding to neutralizing antibodies. It should be remembered that *in vitro* neutralization assays involve a 2-hr incubation of virus with antibody prior to exposure to an environment containing cells carrying CD4 receptors. No such advantage is provided in the *in vivo* neutralization setting.

Fifth, it is possible that appropriate concentrations of antibody permit uptake of virions in the form of immune complexes by Fc receptors on macrophages. These cells support the replication of HIV (22) and may play an important role in early stages of infection, particularly when virus is introduced by mucosal routes. Antibody-mediated enhancement of entry of viruses into macrophages has been partic-

ularly well described in the case of flaviviruses (23–27) and has also been described with retroviruses (28). T-cell lines commonly used for neutralizing antibody titrations lack Fc receptors; thus this phenomenon would not be observed in the course of routine assays. This latter hypothesis has not, however, been supported by our preliminary experiments. HIVIG neutralizing antibody titers were indeed somewhat higher when assays were carried out on mixed cultures of H9 cells and human peripheral blood macrophages, or U937 cells, than when assays were done with H9 cells alone; furthermore, no enhancement of replication was seen with low antibody concentrations (data not shown).

Last, the failure of vaccine trials in which high cell-mediated immunity (CMI) but low neutralizing antibody levels were induced (1–3), together with the present failure of high levels of neutralizing antibody alone to protect, may indicate a necessity for both high levels of CMI and high levels of neutralizing antibody for protection against HIV.

All of the above mechanisms would provide a severe challenge to the development of effective vaccines for the prevention of HIV infection. It should, however, be pointed out that the large number of sexual contacts with HIV-infected persons that are required to induce infection (29) suggests that the infecting dose is usually low, perhaps only one infectious particle. The use of 400 $TCID_{50}$ as a challenge dose in chimpanzee experiments may thus provide an unreasonably severe test for the evaluation of active or passive immunization strategies. Future studies should examine lower HIV challenge doses, as these might provide data more predictive of possible clinical efficacy against low-dose exposures.

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